

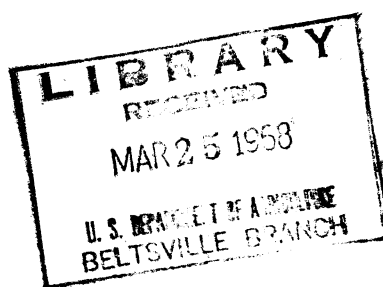
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METHODS OF STUDYING

PLANT HORMONES

and

**GROWTH-REGULATING
SUBSTANCES**



Agriculture Handbook No. 336

METHODS OF STUDYING PLANT HORMONES AND GROWTH-REGULATING SUBSTANCES

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Agriculture Handbook No. 336

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UNITED STATES DEPARTMENT OF AGRICULTURE**

ACKNOWLEDGMENTS

The authors greatly appreciate efforts by all who contributed to this book. The assistance given by the late Paul C. Marth, who was coauthor of the first edition, is gratefully acknowledged.

While it is impracticable to provide a complete list of dealers offering chemicals, apparatus, and materials mentioned in this compilation, a few sources are given to aid the reader in obtaining items that may be difficult to purchase. Trade names and the names of commercial companies are used solely to provide specific information. Mention of a trade product or manufacturer does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture or an endorsement by the Department over other products not mentioned.

This handbook supersedes Agriculture Handbook No. 126,
"Test Methods With Plant-Regulating Chemicals"

Extra care should be exercised in experimenting with new chemicals until it has been determined that they are not harmful to man or animals. The uses of chemicals mentioned in this book are for experimental purposes. These chemicals are not recommended, on the basis of these experimental methods, for application to plants to be used as food.

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METHODS OF STUDYING PLANT HORMONES AND GROWTH-REGULATING SUBSTANCES

INTRODUCTION

The purposes of this compilation are, first, to provide scientists with ready access to methods useful in the study of plant hormones and growth-regulating substances, and, second, as our knowledge in this field advances, to stimulate scientists to devise new methods that will be necessary to understand more fully how hormones and regulators control the behavior of plants.

Information in this handbook is presented in three sections: biological methods that are essential in the study of hormones, some very useful physical methods, and many supplementary techniques that facilitate research in this field. A few very useful methods, such as the well-known oat coleoptile method, have not been included, since these are widely used and descriptions of them are easily obtained.

Methods in this handbook are described step by step in the manner of a cookbook, with the hope that they will be readily understood and easy to use. Descriptive titles have been assigned to facilitate reference to each method.

The index is arranged so methods can be found by approaching the handbook from different viewpoints. For example, from a chemical or biochemical viewpoint, methods useful for detecting gibberellic acid, gibberellin-like compounds, kinetin, or auxin can be readily found. From the viewpoint of the response induced by a hormone or regulator, methods useful in detecting substances that cause cell elongation, abscission, growth retardation, cell division, and other responses can be located. Methods can be found readily that involve the use of specific plant parts or that help accomplish a specific task, such as injecting a regulator into a living cell.

Progress in the accumulation of new scientific knowledge depends upon methods with which the information can be gained. Although the methods described here are useful and serve a definite purpose, new methods must be developed continually to make the most rapid progress in research.

BIOLOGICAL METHODS

ABSCISSION

APPLE PETIOLE METHOD

Based on research by L. J. Edgerton; L. J. Edgerton and M. B. Hoffman.

Description of method—The rate of abscission of petioles of debladed leaves on apple water sprouts following treatment with various chemicals is used as an indication of the effectiveness of the compounds in retarding harvest drop of apples.

This test can also be used to evaluate the effectiveness of plant regulators, other compounds, or combinations of them, in accelerating or reducing the rate of leaf abscission.

Apparatus, Chemicals, and Other Materials

Analytical balance

Hand sprayer, 250 ml., or nasal atomizer, 15–30 ml.

25–200 mg. of each compound to be evaluated
500 mg. of alpha-naphthalene-acetic acid or its sodium salt for standard treatment

Solvent such as 95% ethanol

Wetting agent such as Santomerse, Dreft, or Tween 20

Suggested plant material—Rapidly growing vegetative branches or shoots (commonly called water sprouts) from the main branches or trunks of apple trees (*Malus sylvestris* Mill., also called *Pyrus malus* L.)—McIntosh, Wine-sap, Stayman, or other varieties.

Preparation and selection of plant material—In midsummer tag apple trees that have produced

an abundance of water sprouts during the current season. New sprout growth can be induced to develop 1–2 months earlier than usual by selecting experimental trees before winter pruning and cutting back the existing water sprouts, leaving stubs 3–8 cm. long. These stubs will then produce long, vegetative shoots early during the next growing season. One- to two-year-old potted trees may be used during winter and spring in the greenhouse. Select shoots of uniform diameter, each having 15–25 leaves. Earlier abscission of the control petioles and more uniform results with various test compounds are obtained if the shoots are still growing actively at the time the application is made.

Procedure—Prepare the spray solutions by dissolving the desired weight of the chemical to be evaluated in a minimum of solvent (1–2 ml. of 95% ethanol); add enough wetting agent to make a final concentration of 0.02–0.2% of the wetting agent. Pour this mixture, while stirring, into sufficient tapwater to make a final concentration of 10–30 p.p.m. of the chemical being tested. Apply the spray solution with the hand sprayer to 5 or 6 water sprouts, thoroughly wetting the leaf blades, petioles, and twigs. Within 2–3 days after treatment remove 10 or more leaf blades from each shoot, leaving the entire petioles attached to the twigs. To make the results more nearly uniform, do not use the 4 or 5 basal leaves or the young, partially expanded leaves near the tip of the shoots. Remove blades from 10 or more leaves on comparable unsprayed sprouts of each test tree and designate these as controls.

Method of taking results—Within 10–11 days after treatment, and at frequent intervals thereafter for the duration of the test, count the petioles that remain attached in each experimental lot. The final percentage of petioles attached is an index of the effectiveness of the compound when used for retarding fruit abscission.

Suggested standard for comparison—10, 20, or 30 p.p.m. concentration of alpha-naphthalene-acetic acid or its sodium salt.

References

- Edgerton, L. J. A Method for Evaluating the Effectiveness of Growth Substances in Delaying Apple Abscission. *Amer. Soc. Hort. Sci. Proc.* 49: 42–44. 1947.
 Edgerton, L. J., and Hoffman, M. B. The Effect of Some Growth Substances on Leaf Petiole Abscission and Preharvest Fruit Drop of Several Apple Varieties. *Amer. Soc. Hort. Sci. Proc.* 62: 159–166. 1953.

ATTACHED PETIOLE METHOD

Based on research by J. W. Mitchell, P. B. Marsh, and Robert Bender.

Description of method—The test compound is placed on debladed petioles, and a known pressure is then applied to the petioles each day to determine the length of time required for the petioles to abscise compared with that for untreated petioles.

Apparatus, Chemicals, and Other Materials

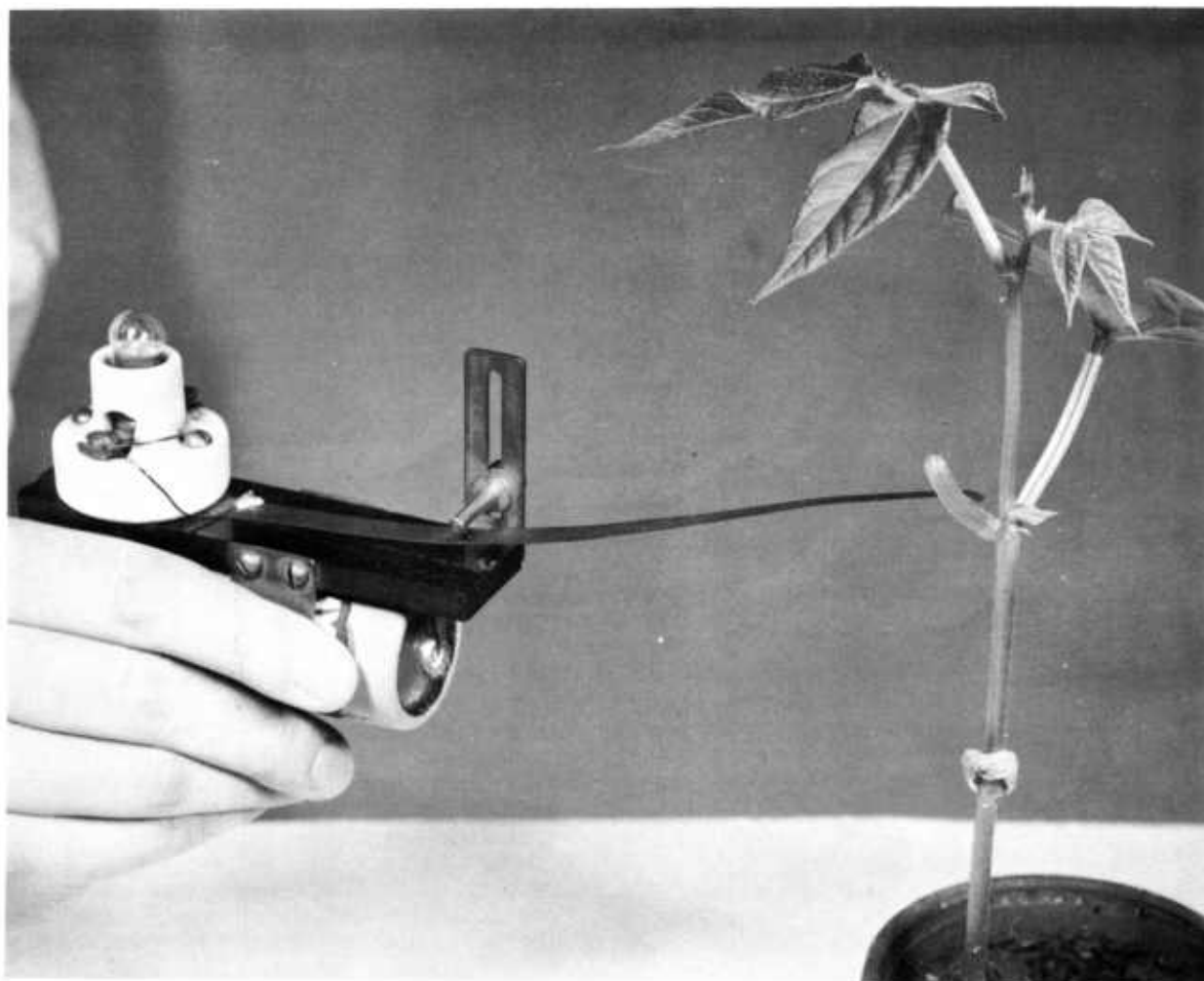
Constant-pressure apparatus (fig. 1)
 Razor blades or sharp knife
 Lanolin
 1–2 g. endothal acid, monohydrate
 1–2 g. phenylmercuric acetate
 Analytical balance
 Vials
 Medical applicator sticks or toothpicks
 Tween 20
 Compounds to be evaluated as growth regulators

Suggested plant material—Young cotton plants (*Gossypium hirsutum* L.)—Deltapine variety, and young bean plants (*Phaseolus vulgaris* L.) of the Pinto or other varieties.

Preparation and selection of plant material—Plant cotton seeds in composted soil contained in small clay pots, using several seeds per pot. After the plants have developed cotyledons about 4 cm. across, and the first true leaf is beginning to expand from the terminal bud (10–12 days after planting), select a group of test plants of uniform size and thin them to one plant per pot. Arrange the plants uniformly in rows of 5 plants each on a greenhouse bench that is evenly illuminated. Remove the blade of one cotyledon of each plant by severing the petiole where it joins the blade.

Procedure—Dissolve 12.5 mg. of the compound to be tested in approximately 0.25 g. of Tween 20 (7 drops from a 10 ml. pipette); then add 1 g. of melted lanolin. Stir the mixture thoroughly. Dilute one-half of this 1% mixture with an equal amount of the Tween 20-lanolin carrier, and continue the dilution process with the mixture obtained until a suitable range of dosage levels has been obtained.

Treat the debladed petioles of one row of plants by applying with disposable applicator sticks a portion of the 1% mixture about the size of a wheat seed as a band about 2 mm. wide around the cotyledonary stump of each plant. Apply the paste 2–3 mm. from the stems. Apply



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FIGURE 1.—Pressure applicator used to determine whether chemical will make leaves fall from plant by applying measured amount of pressure to both treated and untreated petioles.

equal portions of the pastes containing progressively smaller amounts of the test compound to debladed petioles in corresponding rows in a similar manner. Apply an equal amount of the lanolin-Tween 20 mixture without a chemical added to one more row of petioles and designate these as treated controls.

To determine when the petioles readily abscise, apply pressure once each day in a downward direction against the upper surface of each debladed cotyledonary petiole. Accomplish this by pressing the thin metal spring of the pressure applicator against the petiole until tension equal to approximately 5 g. develops, at which time the light on the tension applicator will flash (fig. 1). Always apply pressure to the petiole at a predetermined distance (8–10 mm.) from the stem. The pressure applicator may be

calibrated prior to use by pressing the spring on a balance with a 5-g. weight on the opposite pan.

Method of taking results—Record the number of petioles that abscise each day and calculate the number that fall from the treated plants in terms of the number that fall from plants treated with the Tween 20-lanolin carrier alone.

For an alternate method, use young bean plants in the same manner except remove one primary leaf blade of each plant, leaving the petiole attached. Select plants with primary leaves that are approximately 3–5 cm. wide, and with the first trifoliate leaf just beginning to unfold from the terminal bud.

Suggested standard for comparison—Endothal acid monohydrate, phenylmercuric acetate, or

naphthyl maleimide for cotton plants; endothal acid monohydrate or phenylmercuric acetate for bean plants.

Reference

Mitchell, J. W., Marsh, P. B., and Bender, Robert. [Unpublished data.]

COLEUS PETIOLE METHOD

Based on research by L. C. Luckwill.

Description of method—Small filter paper disks impregnated with the compound being tested are applied to the cut ends of petioles of excised nodes of coleus. Delay in abscission due to the presence of the chemical is measured by comparing the time required for treated and untreated petioles to abscise. Within limits, this delay in abscission is proportional to the logarithm of the quantity of growth substance applied. The method is believed to be specific for auxins, with the possible exception of kinetin, and it is particularly convenient for the detection of auxins on paper chromatograms. Under some conditions this method can be used to detect growth-accelerating substances even in the presence of inhibitors that would, in other methods, mask the accelerating effects.

Apparatus, Chemicals, and Other Materials

Analytical balance
Razor blade
Filter paper
Sheets of glass
Dishes larger than the sheets of glass
Incubator maintained at 25° C.
Needle attached to wooden handle
Disks of filter paper, Whatman No. 1, 0.5 cm. in diameter, made with a paper punch
Straight pins
Modeling clay
Pipette, 0.1 ml., graduated in 0.01-ml. divisions
Microsyringe
Test tubes
Corks to fit test tubes, or Parafilm
50 mg. of compound to be tested
0.25 g. of alpha-naphthaleneacetic or indole-3-acetic acid for standard treatment
Distilled water

Suggested plant material—Coleus (*Coleus blumei* Benth.)—green-leaved variety Corunna; or other ornamental forms.

Preparation and selection of plant material—Grow the plants with a moderate or low light

intensity and short days so that long internodes are developed. Avoid excessive nitrogen fertilization since this delays petiole abscission. Use controlled growth conditions to obtain the most uniform experimental plants. Allow the plants to grow until shoots about 30 cm. long with 8 pairs of expanded leaves can be obtained.

Procedure—With a razor blade, cut the required number of vegetative shoots, each about 30 cm. long. Use only 4–7 pairs of leaves (counting from the apex) for the experiments. Excise the 4–7 nodes with attached leaves by severing the stem 1 cm. above and below each node (fig. 2, A). Remove the laminae and axillary buds. Cut each petiole in a plane parallel to and at a point 4 mm. from the stem (fig. 2, B). Split the stem piece vertically (fig. 2, C). Place the 2 half-stems with their attached petiolar stubs on moist filter paper (fig. 2, D) supported on glass in a dish. Impale one of the disks of filter paper on the point of a pin and support the pin in an inverted position by pressing the head into a block of modeling clay. Impregnate the disk with the compound to be tested by adding to each disk a measured drop of alcoholic solution delivered from a microsyringe. The alcohol should contain a measured amount of the compound being tested. Dry the disks, place them in corked tubes, and store them in a cool, dark place. Just before applying the disks to the petiole, dip them momentarily, and one at a time into distilled water; or support them on a pin and add to each one slightly more than the amount of water required to saturate the disk, using a 0.1-ml. pipette graduated in 0.01-ml. divisions. Immediately place the disks in contact with the freshly cut surface of

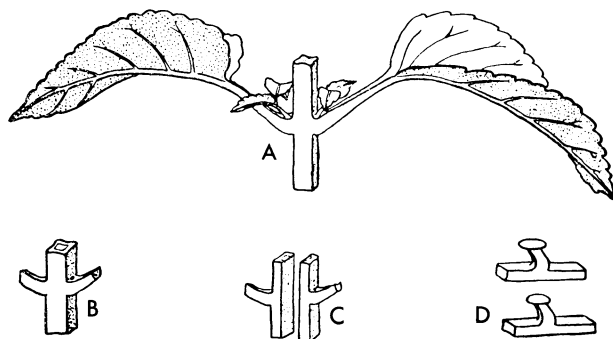


FIGURE 2.—Preparing coleus nodes to determine effect of regulating substances on abscission. Steps include: A, Node with attached leaves; B, axillary buds removed, petioles cut to 4 mm.; C, stem piece split vertically; D, filter paper disk containing auxin or plant extract applied to cut surface of petiolar stump. (Diagram—courtesy of Journal of Horticultural Science.)

the petiolar stump (fig. 2 D). After the required number of petioles have been treated, cover the dish with a sheet of glass with a moist sheet of filter paper on the underside to maintain relatively high humidity within the chamber. Store the dish containing the explants in darkness at 25° C.

This method is particularly well adapted for detecting and measuring auxins on paper chromatograms. To utilize the test in this way, use a paper punch to punch out disks 0.5 cm. in diameter along the length of the chromatogram and record the R_f position of each disk. Moisten the disks individually with the required amount of distilled water using pins and the method described above. Immediately place each disk separately on one of the coleus petioles, prepared as described above. If a single chromatogram contains insufficient auxin to give a response, place 2 or more disks, punched from comparable positions on an adjacent chromatogram, on a single petiole.

Method of taking results—Record daily the number of petioles that abscise after pressing each petiole lightly with a needle or apply a uniform pressure to determine whether abscission has occurred. (See "Attached petiole method," p. 2.)

Suggested standard for comparison—Alphanaphthaleneacetic acid, 0.1–1.0 microgram per petiole.

Reference

Luckwill, L. C. Two Methods for the Bioassay of Auxins in the Presence of Growth Inhibitors. *Jour. Hort. Sci.* (London) 31: 89–98. 1956.

EXPLANT METHOD

Based on research by F. T. Addicott, R. S. Lynch, G. A. Livingston, and J. K. Hunter; variations by F. T. Addicott, R. S. Lynch, G. A. Livingston, and J. K. Hunter; and by F. T. Addicott, H. R. Carns, J. L. Lyon, O. E. Smith, and J. L. McMeans.

Description of method—Small segments of leaves (explants) containing an abscission zone are treated with compounds, and the effect on abscission is observed.

Apparatus, Chemicals, and Other Materials

Scalpel
Forceps
Filter paper
Petri dishes

Dark chamber maintained at about 25° C.

Analytical balance

Closed bell jar or desiccator

0.5–1 g. of each compound to be evaluated

For standard treatment:

Ethylene gas or 0.5–1 g. indole-3-acetic acid

Agar

Suggested plant material—*Citrus* spp. leaves or leaves of other kinds of plants that have abscission zones near a leaf blade.

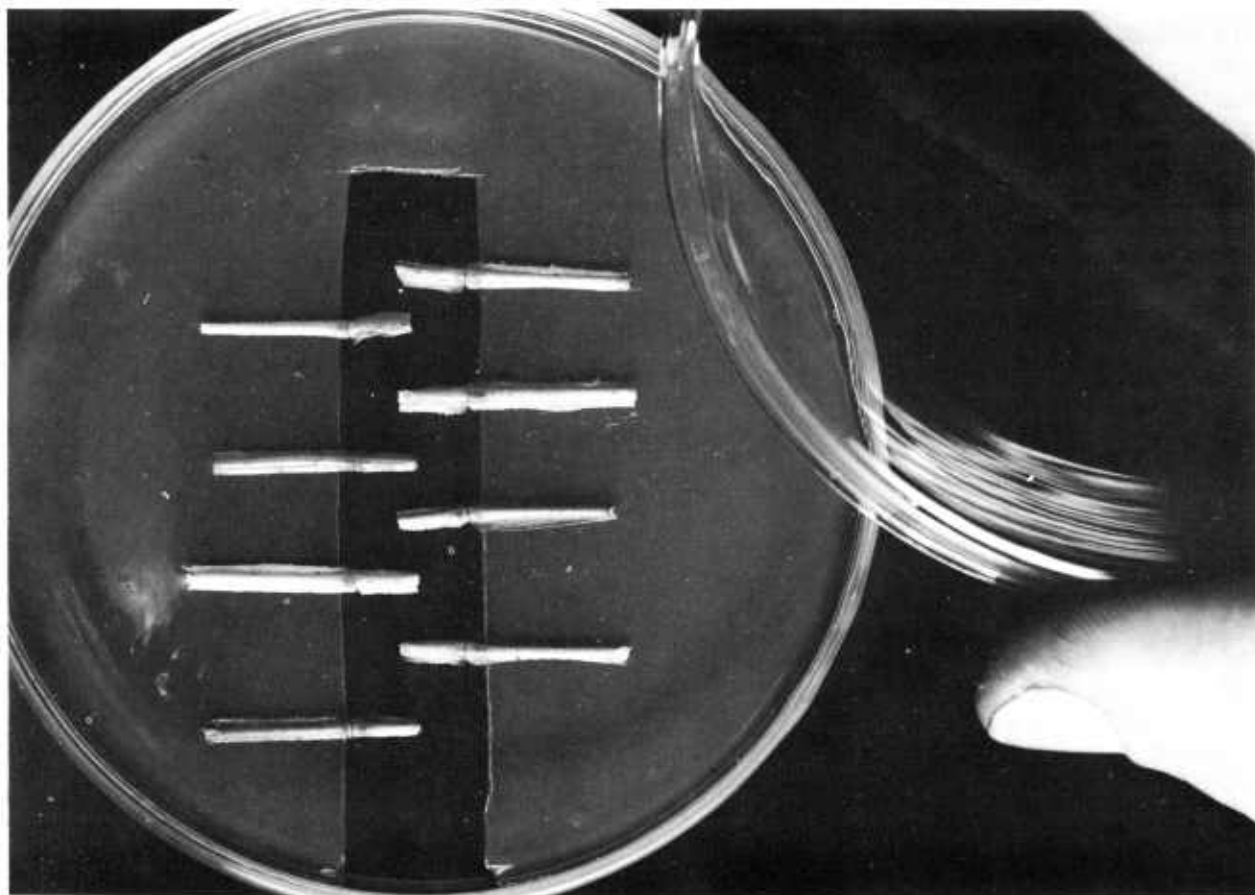
Preparation and selection of plant material—Excise for use as test material segments of leaves containing the abscission zone and the adjacent tissues (fig. 3). These segments are called explants. Trim off all but the midrib of the blade portion of the leaves in preparing the explants. If the petioles are winged, trim the wing from them. Always leave a longer piece of tissue, consistently either distal or proximal to the abscission zone, so that identification of the blade or petiole tissue can be made readily. Prepare 10 of these explants for each concentration of each treatment, and an additional 20 or 40 to serve as controls. After cutting, and before treatment, store the explants on moist paper in a petri dish. In preparing the explants, utilize leaves that have attained their full size and hard texture but still are dark green in color.

Procedure—Prepare 4% agar in water, pour it into petri dishes, and allow it to cool and set. The agar in the dish should be about 6 mm. deep. After the agar has set, remove a strip of it, about 3 cm. wide, along the diameter of the plate, leaving a narrow bridge of agar across one end to hold the agar in place and to act as a marker (fig. 3).

Prepare 10–25 ml. of each concentration of each chemical to be tested. The suggested concentration range is 1–1,000 p.p.m. Treat explants (10 per treatment) by immersing them in the test solution for 30–60 seconds. Remove the treated explants from the solution and mount them on the agar of a petri dish so the shorter part and the abscission zone overhang the central channel cut in the agar. The compound may also be administered by including it in the agar support or by applying small droplets to the cut petiole or blade surface of the explant.

Prepare dishes containing untreated explants to serve as controls. Keep the petri dishes containing the explants in the dark at about 25° C. except for the short daily period of examination.

To use a somewhat different method of supporting the explants, construct a holder by supporting a Pyrex glass rod horizontally on a



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FIGURE 3.—Test used to study physiology of abscission: Treated and untreated segments of leaves, each including an abscission zone, are placed on agar in petri dishes and the rate of abscission is observed.

curved foot at each end of the rod. Add a row of fine glass pins projecting from each side of the rod (fig. 4). Place the holder in a petri dish and pour 2% agar around the feet of the holder to prevent displacement and to maintain a high relative humidity within the closed petri dish. Mount the explants on the holder by forcing the cut end of each explant over one of the glass pins.

Method of taking results—Observe for evidence of abscission by examining the explants with the dishes closed. To determine if abscission is complete but not evident, raise the dish cover and lift the explants slightly by pressing the tips of forceps upward under their free ends. The period during which the explants are kept under observation is usually 6 or 7 days. With some experience, various “stages” of abscission can be identified. Maintain daily records, listing

the percentage of abscission in each treatment and, if desired, the “stage” of abscission that each explant exhibits at each time of observation.

If the objective of any test or series of tests is to determine the effectiveness of compounds in producing the abscission of organs, the only fully reliable tests are those conducted with whole plants. Furthermore, if the compounds are being tested for potential field use, testing under a variety of field environmental conditions is essential.

Suggested standard for comparison—To initiate or accelerate abscission, place petri dishes containing the explants inside an airtight container and add ethylene to make a concentration of 20–50 p.p.m. To delay abscission, use 0.001–100 p.p.m. of indole-3-acetic acid (30–60 seconds dip) as a standard.

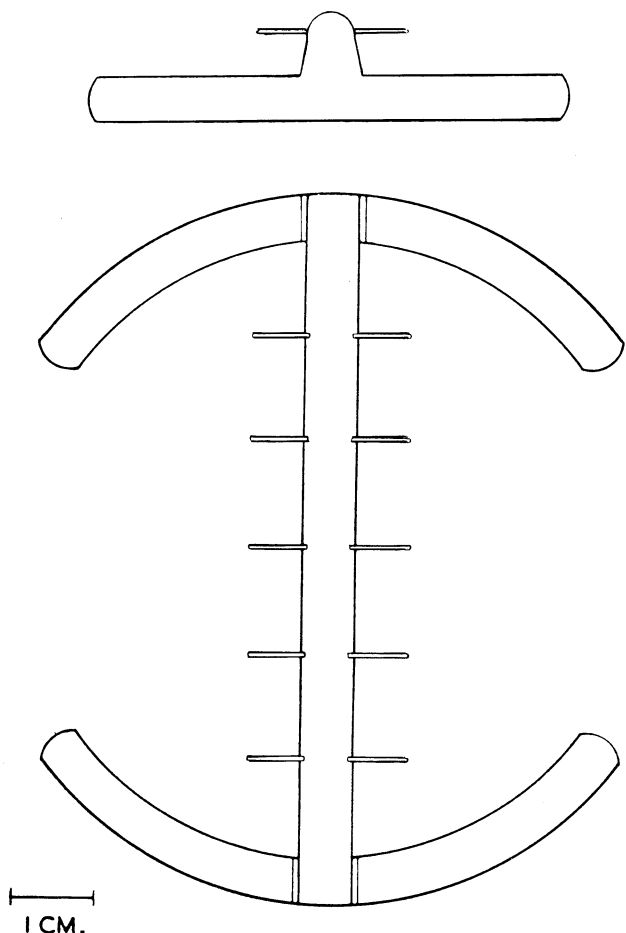


FIGURE 4.—Pyrex glass explant holder, 2 views. (Photo—courtesy of Plant Physiology.)

Variation used by Addicott, Carns, Lyon, Smith, and McMeans—Another variation, involving the use of young cotton plants, is shown diagrammatically in fig. 5. Using strips of sheet stainless steel, form legs designed to raise the main part of the strip above the bottom of the petri dish. Drill 2 rows of 5 holes each in the horizontal part of the strip. Place the stainless steel explant holder in the dish. Excise cotyledonary nodes from cotton seedlings 2 or more weeks old. Make explants consisting of two 3-mm. petiole stumps with a 3-mm. stem stump and 10 mm. of hypocotyl attached. Place these in the stainless steel holders in the petri dishes. Apply the test chemical mixed in 1% agar as 5-microliter droplets to the cut surface of the petioles. During the next 4 days apply daily a known pressure to each petiole stump using an abscissor described by Addicott, Lynch, Livingston, and Hunter (paper cited below); or

apply a known pressure using a pressure applicator. (See "Attached petiole method," p. 2.) Record the number of petioles that abscise.

References

- Addicott, F. T., Lynch, R. S., Livingston, G. A., and Hunter, J. K. A Method for the Study of Foliar Abscission In Vitro. *Plant Physiol.* 24: 537–539. 1949.
Addicott, F. T., Carns, H. R., Lyon, J. L., Smith, O. E., and McMeans, J. L. On the Physiology of Abscissions. *Régulateurs Naturels de la Croissance Végétale* 123: 687–703. 1963.

LANOLIN PASTE METHOD

Based on research by P. C. Marth, W. H. Preston, Jr., and J. W. Mitchell.

Description of method—The abscission of apple fruits following application of a chemical in lanolin paste around the stem of the individual fruits before the usual harvest date indicates the effectiveness of the chemical in retarding fruit abscission.

With slight modifications this test is applicable to studies of abscission rates of a wide variety of fruits.

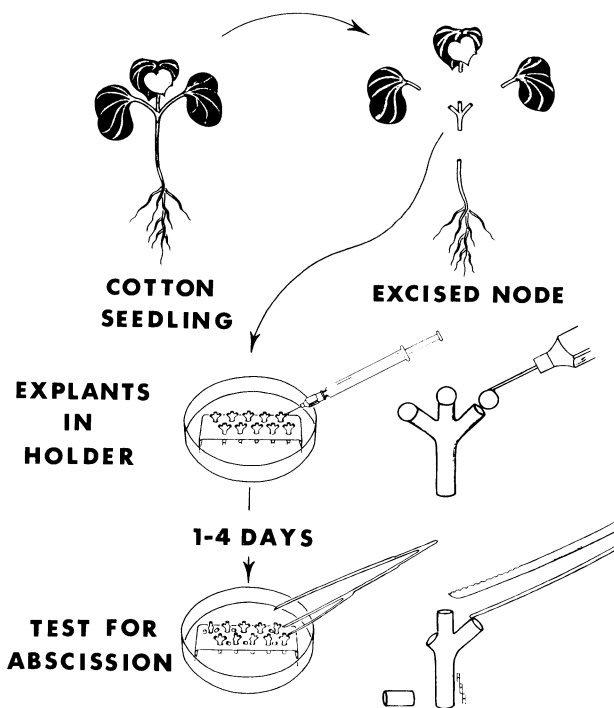


FIGURE 5.—Variation of explant method using cotton plants to investigate abscission. (Diagram—courtesy of *Régulateurs Naturels de la Croissance Végétale*.)

Apparatus, Chemicals, and Other Materials

Analytical balance
 Small shell vials with stoppers
 Wooden board about 15 cm. wide \times 30–60 cm. long \times 3 cm. thick having 1-cm. diameter holes drilled 2 cm. deep and 5 cm. apart for carrying the vials in the field
 Toothpicks or disposable wooden applicators without cotton
 Small labels with string or wire attached
 25–100 mg. of each compound to be evaluated
 0.5 g. of alpha-naphthaleneacetic acid for standard treatment
 Ethanol
 Lanolin
 Tween 20

Suggested plant material—Apple trees (*Malus sylvestris* Mill., also called *Pyrus malus* L.) bearing an abundance of fruit readily accessible from the ground—Grimes, McIntosh, York, or other varieties.

Preparation and selection of plant material—Select individual fruits that are readily accessible from the ground and located around the perimeter of each tree used for the experiment. The fruits should be of uniform size and free of blemishes. Make the fruit selection and apply the treatments about 30 days before the estimated, or usual, harvest date for the variety.

Procedure—Prepare lanolin-Tween 20 mixtures containing 0.1 and 1% concentrations of the respective compounds. To prepare the 1% mixture, place 25 mg. of the compound to be tested in a vial and add 14 drops of Tween 20. Stir to dissolve the chemical, and add 2 g. of lanolin. Melt the lanolin by placing the vial in warm water (not over 55° C.) for a few minutes. Remove the vial and stir the mixture thor-

oughly until it reaches room temperature and becomes semisolid. To prepare the 0.1% mixture, weigh 0.2 g. of the 1% mixture previously prepared and mix this with 1.8 g. of the lanolin-Tween 20 carrier (the carrier made by mixing 2 g. of lanolin with 14 drops of Tween 20).

Apply a small portion, the size of a wheat seed, of the mixture as a band about 3 mm. wide around the stem of each of 10 fruits near the junction of stem and branch. As each fruit is treated, attach a label designating the treatment. Repeat, using an additional 10 fruits for each mixture. Fruits used for an individual mixture should be distributed evenly around the perimeter of the tree. Remove all untreated fruits except 10 that are left as controls.

Method of taking results—One week after treatment and at weekly or biweekly intervals thereafter (depending upon the prevalence of wind or hail), record the number of fruits remaining attached in each treatment. Record data regarding advance or retardation in maturity, color changes, cracking, or other responses for 30 days or more after the usual harvest date for the variety. The percentage of treated fruits remaining attached throughout the course of the experiment compared with the percentage of untreated fruit remaining attached serves as a measure for evaluating the effect of individual compounds on fruit abscission.

Suggested standard for comparison—Lanolin paste mixture containing either 0.1 or 1% alpha-naphthaleneacetic acid.

Reference

Marth, P. C., Preston, W. H., Jr., and Mitchell, J. W. Relative Effectiveness of the Mono-, Di-, and Tri-Chlorophenoxyacetic Acids in Retarding the Abscission of Mature Apples. Bot. Gaz. 117: 51–55. 1955.

ABSORPTION AND TRANSLOCATION

AGAR BLOCK POLAR TRANSPORT METHOD

Based on research by J. B. Zaerr. (Original method by F. W. Went and H. G. van der Weij.)

Description of method—A segment of stem, branch, or petiole is placed either upright or in an inverted position on a receiver block of agar. A donor block of agar containing a known amount of radioactively tagged regulator is then placed on the upper end of the segment. After an appropriate period of time, the amount of radioactivity translocated from the donor to the receiver block is determined by extracting

the receiver block and measuring the amount of radioactivity obtained. Identity of the translocated material is established chromatographically. Polar transport is measured by comparing the amount of growth regulator translocated from the donor to the receiver block when the segment is upright with respect to its usual position of growth and when it is inverted with respect to this position.

Apparatus, Chemicals, and Other Materials

Clay pots, 7.5 cm.
 Composted soil
 Common cutter that can be adjusted to cut

5-mm. and 2-mm. segments (See "Segment cutters," p. 101.)
Forceps for handling segments
Glass microscope slides
Spatula for manipulating agar blocks
Petri dishes
Glass tube, 15–30 cm. long, 5 mm. inside diameter
Solid glass rod, about 30 cm. long, 4 mm. diameter
Filter paper
Aluminum foil
Paper chromatography equipment
Constant temperature chamber—24°–27° C.
X-ray (no-screen type) film for autoradiographs of chromatographed extracts
Glass vials for extracting agar blocks, 1–2 ml. capacity
Concentric ring planchets for measuring radioactivity
Disposable pipettes for handling agar extracts
Gas flow counter
Scaler
Analytical balance
Radioactively tagged compounds to be evaluated
95% ethanol
Isopropanol, ammonia, or other solvents suitable for chromatographing the growth regulators involved
Agar (ionagar)
Indole-3-acetic-1-C¹⁴ acid for standard treatment

Suggested plant material—Segments of petioles, internodes, or hypocotyls obtained from young succulent plants, such as bean (*Phaseolus vulgaris* L.), cucumber (*Cucumis sativus* L.), and tomato (*Lycopersicon esculentum* Mill.).

Preparation and selection of plant material—Using bean stem segments as an example of test plant material, sow 3–5 seeds in each of 36 clay pots containing composted soil. Moisten the soil and germinate the seeds. When the primary leaves are still wrinkled and the terminal bud is 2–3 mm. long, select a group of plants with straight hypocotyls of uniform size and stage of development. Cut 5-mm. segments, one from each hypocotyl, beginning 5 mm. below the cotyledons. Use the segments immediately.

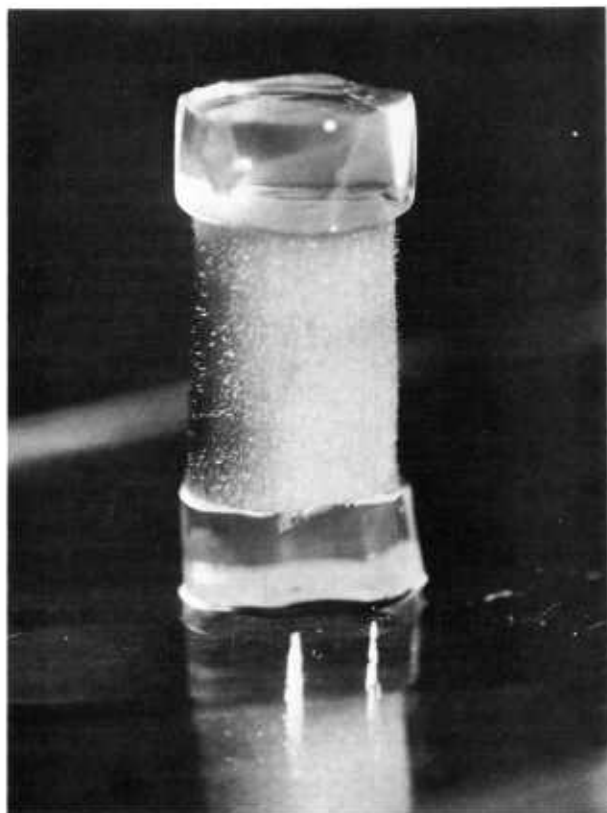
Procedure—Make 1% agar by mixing together and bringing to a boil 0.1 g. of the agar and 10 ml. of water. Pipette this hot agar into the long glass tube. Hold a finger over the open lower end to keep the agar within the tube. When the tube is full, place another finger over

the upper end of the tube and hold the entire tube under a stream of cold running water. When the agar has solidified (1–2 minutes), tap the tube endwise gently on a hard surface to loosen the agar from the inside wall. Force the agar from the tube with the solid glass rod. Repeat this process until sufficient molded agar is obtained. Cut the agar rods into cylindrical blocks 2 mm. long using the cutter. (See "Segment cutters," p. 101.) Place agar blocks on a glass slide inside a petri dish lined with moist filter paper. Store at 5° C. until used. To prepare donor blocks, soak plain agar blocks overnight in an aqueous solution of the growth regulator to be tested. Remove these blocks from the solution and store as described above.

Place a receiver agar block (plain block) on a glass microscope slide. Cut a 5-mm. segment from the plant and place this segment upright (lower cut surface down) on the receiver block. Place a donor block (containing the growth regulator) on the upper cut surface (fig. 6). Repeat this procedure, inverting the plant segment (apical end resting on the receiver block and basal end supporting the donor block). Replicate both positions as many times as desired. Place the glass slides in petri dishes lined with moist filter paper, wrap the dishes in aluminum foil, and store at a constant temperature of about 25° C. for the desired transport period (the time interval during which segments are in contact with the donor and receiver blocks), which varies from several hours to several days, depending upon the growth regulator involved.

Method of taking results—At the end of the transport period, remove and discard the donor blocks and plant segments. Extract each receiver block separately, using 0.3 ml. of distilled water. Soak each block at least ½ hr. for each extraction and extract 4 times, using fresh water each time. Combine the extracts from each block, place each combined extract on a concentric ring planchet, and evaporate to dryness. Measure the radioactivity present on each planchet with a gas-flow counter. Compare the amount of radioactivity that passed through upright segments with the quantity that passed through inverted segments. Compare the substance collected in the receiver blocks with the substance initially contained in the donor blocks by means of autoradiographs of chromatographed extracts from donor and receiver blocks.

Suggested standard for comparison—Indole-3-acetic-1-C¹⁴ acid, specific activity about 30 microruries per micromole, concentration 1 mg./liter, transport period 8 hr.



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FIGURE 6.—Hypocotyl segment used to measure polar transport showing agar blocks in place: Upper, donor block containing regulator; lower, receiver block with no regulator.

Reference

Method submitted by J. B. Zaerr, Forest Research Laboratory, Oregon State University, Corvallis. See also "Agar block preparation and use," p. 96.

BEAN ROOT METHOD

Based on research by T. J. Muzik.

Description of method—Test plants are arranged with one-half of their root systems in the test solution and the other half in tapwater; then the roots that have been in the test solution are cut off at different time intervals. The resulting growth modifications or injurious effects of the chemicals can be observed, and the rapidity of absorption and translocation of the test compound by the roots can be determined.

This method can be used to study the effects of environmental factors (light, tempera-

ture, hydrogen ion concentration, mineral nutrition, and oxygen supply) on the ability of roots to absorb and translocate regulating chemicals.

Apparatus, Chemicals, and Other Materials

Clay pots, 8–15 cm.

Composted soil

Glass containers, 200–500 ml.

Supports to hold a plant with one-half of its root system in one container, the other half in another container

Analytical balance

Compressed air for aerating nutrient solutions

Greenhouse space with controlled temperature, humidity, and light intensity, or with devices for recording temperature, humidity, and light intensity

2–5 g. of each compound to be evaluated

3-(para-chlorophenyl)-1,1-dimethylurea for standard treatment

For nutrient solution:

124 g. of $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$

83 g. of $\text{CaCl}_2 \cdot 6 \text{H}_2\text{O}$

68 g. of KH_2PO_4

85 g. of $\text{Ca}(\text{NO}_3)_2$

Preparation and selection of plant material—

Grow bean plants in soil or other suitable medium. After the plants have developed one partly expanded trifoliate leaf, select the desired number of plants of uniform size and carefully wash them free of soil. Support the plants with their roots in nutrient solution aerated by compressed air, and allow them to grow 3–5 days to condition them for the test. For the nutrient solution, use stock solutions of MgSO_4 , CaCl_2 , KH_2PO_4 , and $\text{Ca}(\text{NO}_3)_2$ made by dissolving the quantities listed above, each in a separate liter of tapwater. To 500 ml. of tapwater add 9 ml. each of the MgSO_4 , CaCl_2 , and KH_2PO_4 stock solutions, and 18 ml. of the $\text{Ca}(\text{NO}_3)_2$ stock solution. Add sufficient water to make 1 liter of nutrient solution.

Suggested plant material—Plants, such as beans (*Phaseolus vulgaris* L.), with root systems that can be arranged in two portions of about the same size.

Procedure—Arrange pairs of containers for the test plants and fill one container of each pair with a water solution of the compound to be tested (25–200 p.p.m.). Fill the remaining container of each pair with tapwater. For the control plants fill both containers with tapwater.

Select plants of uniform size and vigor from those placed in nutrient solution earlier. Support each test plant over a pair of containers and arrange the roots so that one-half of the

root system is immersed in the test solution and one-half in tapwater. Arrange control plants with each half of the root system growing in tapwater. In this manner, use 6–12 plants for each test solution and an equal number of plants with their roots in tapwater for untreated controls. Allow the plants to remain in a greenhouse and record the prevailing temperature, humidity, and light intensity, or grow them under controlled conditions.

After 30 minutes, cut from one-third of the treated plants the roots immersed in the test solution but leave on these plants the roots growing in tapwater. Also cut one-half of the roots from each of one-third of the control plants. After another 30 minutes repeat this procedure, using the second third of the treated and control plants. Finally, repeat the procedure after an additional 30 minutes, using the remaining treated and control plants. Replace the tapwater with nutrient solution that is continuously aerated, and allow the treated and untreated plants to grow for a week or two with their remaining roots in the nutrient solution.

Method of taking results—Epinasty, formative effects (leaf modification), discoloration of leaves, or gall formation observed on the treated plants, but not on untreated controls, indicate absorption and translocation of the test compound. In addition, the time required for this to occur through the roots can be determined by comparing the treated and untreated plants that have had their roots removed at different intervals.

Suggested standard for comparison—25–200 p.p.m. of 3-(para-chlorophenyl)-1,1-dimethylurea.

Reference

Unpublished method submitted by T. J. Muzik, Department of Agronomy, Agricultural Experiment Station, Washington State University, Pullman.

BEAN STEM CURVATURE METHOD

Based on research by A. S. Crafts; B. E. Day; J. W. Mitchell, W. M. Dugger, Jr., and H. G. Gauch.

Description of method—The compound to be tested is placed on one leaf of a plant; then absorption and translocation of the compound (or of a metabolite of it) are evaluated on the basis of stem curvature that develops. This test is applicable only to compounds that induce or retard cell elongation.

The test can be used under controlled conditions to compare the relative effectiveness of different compounds that are known to cause stem curvatures. With some modifications the test can also be used to detect the effects of adjuvants on absorption and translocation of a regulator, or metabolite of it, that induces stem curvature at a distance from the area to which the regulator is applied.

Apparatus, Chemicals, and Other Materials

Clay pots, 8–15 cm.
Composted soil
Greenhouse space maintained at about 24°–29° C.
Analytical balance
Several 0.1-ml. pipettes graduated in 0.01-ml. divisions
Beakers for preparing solutions, 250 ml.
Glass applicator (about 15 cm. long) with one end drawn out to make a narrow portion of about 2-mm. diameter and 7-cm. length
Facial tissue or paper towels
Source of fluorescent white light
Protractor
Adjustable bevel square
About 100 mg. of each compound to be evaluated
100 mg. of 2,4-dichlorophenoxyacetic acid for standard treatment
Tween 20, Glim, Joy, or other solvent of the liquid detergent type
Distilled water

Suggested plant material—Young bean plants (*Phaseolus vulgaris* L.)—Black Valentine, Red Kidney, Pinto, or other varieties that germinate and grow uniformly.

Preparation and selection of plant material

Plant 3 or 4 seeds per pot in composted soil. After the plants have developed partially expanded but still wrinkled primary leaves, and the trifoliate leaves are still tightly folded in the terminal buds, select one plant in each pot for similarity of size. Sever the stems of the remaining plants in each pot at the soil level and discard them. Place the selected plants in a well-lighted greenhouse with the pots widely spaced so that the plants will receive adequate illumination. As soon as the primary leaves of the majority of the plants have expanded and are no longer wrinkled, usually about 48 hr. after the first selection, reselect the plants for size and uniformity, and place them in rows of 8–10 plants each. Water the soil surrounding the roots of each plant before conducting the test.

Procedure—Dissolve in Tween 20 or other suitable solvent a weighed portion of the compound to be tested and add this mixture to warm, distilled water so 0.02 ml. of the final mixture will contain 1–5 μ g. of the chemical and sufficient Tween 20 to make a final concentration of 0.1% of this solvent.

Fill a pipette with the solution; then drain exactly 0.02 ml. out so that it adheres as a drop on the tip of the pipette. Touch the tip of the pipette near one edge of one primary leaf blade and move the tip across the leaf, thus applying the mixture as a narrow band extending from one side of the leaf blade to the other (fig. 7, *left*). Immediately support the blade by pressing a folded pad of facial tissue or paper towel under the leaf, place the applicator in the mixture, and move the applicator slowly toward the tip of the leaf, thus spreading the liquid evenly over the surface of the leaf blade (except for an area near the end to which the petiole is attached (fig. 7, *right*). Apply other 0.02-ml. portions to one primary leaf of each of the remaining plants in the row.

Make certain that the treated leaves are oriented on the same side of each row. Arrange as controls a comparable row of untreated plants, and a third row treated similarly with water and Tween 20, or other solvent. Allow the plants (8–10 per treatment) to grow in a

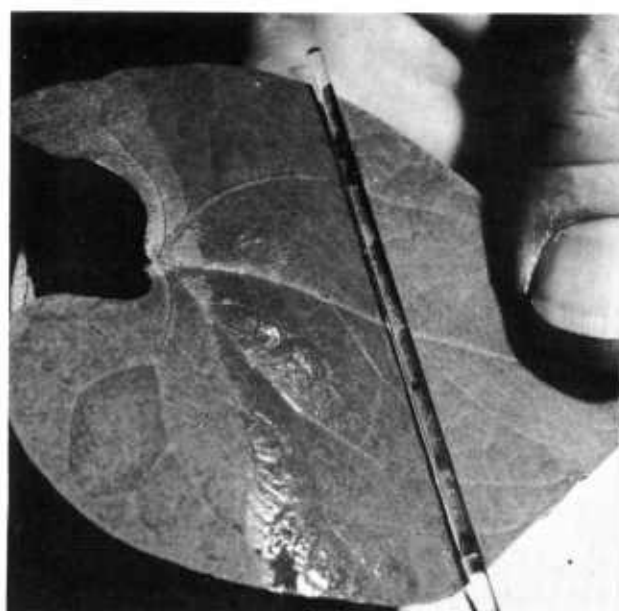
greenhouse, or place them in the fluorescent light so they receive illumination of at least 700-foot-candle intensity.

Method of taking results—Observe the plants at 30-minute or hourly intervals. Measure the angle of stem curvature resulting from the presence of the chemical by lining up one leg of the adjustable square with the hypocotyl and lower half of the first internode, and adjusting the other leg of the square so that it is in line with the upper half of the first internode and second internode above the curved section of stem. Compare average readings from the control plants with those from plants that received the compounds being tested.

Suggested standard for comparison—1.0 μ g. of 2,4-dichlorophenoxyacetic acid on one primary leaf of each of 8–10 plants.

References

- Crafts, A. S. Herbicides, Their Absorption and Translocation. *Agr. and Food Chem.* 1: 51–55. 1953.
Day, B. E. The Absorption and Translocation of 2,4-Dichlorophenoxyacetic Acid by Bean Plants. *Plant Physiol.* 27: 143–152. 1952.
Mitchell, J. W., Dugger, W. M., Jr., and Gauch, H. G. Increased Translocation of Plant Growth-Modifying Substances Due to Application of Boron. *Science* 118: 354–355. 1953.



PN-294, A and B

FIGURE 7.—Applying minute, measured amount of growth-regulating chemicals to leaves: *Left*, applying with a pipette a measured volume of mixture as a band across the upper surface of young leaf; *right*, spreading mixture evenly over upper surface of leaf with a glass rod applicator.

ANGLE AND ELONGATION OF BRANCHES

LANOLIN METHOD

Based on research by A. P. Preston and H. W. B. Barlow; Leif Verner.

Description of method—A piece of flexible tubing is half filled with paste containing the test chemical and slipped onto the stump of a decapitated branch of a young tree. The effects on branch-trunk angle and growth of the branch are noted.

Apparatus, Chemicals, and Other Materials

Analytical balance
About 1 m. of 6-mm. flexible rubber tubing or sheets of thin, transparent celluloid
Protractor
Vials
1 g. of each compound to be evaluated
About 2 g. of 3-indolebutyric acid for standard treatment
Lanolin

Suggested plant material—Young trees in whip stage (no lateral branches) such as apple (*Malus sylvestris* Mill., also called *Pyrus malus* L.), pear (*Pyrus communis* L.), plum (*Prunus* spp.), willow (*Salix* spp.).

Preparation and selection of plant material—Select trees of uniform size and stage of development. Use 10 trees for each compound or concentration to be tested.

Procedure—Prepare lanolin mixtures, 3 g. of lanolin per mixture, containing an amount of the chemicals to be tested covering a range of 0.1–1% of the weight of lanolin used. Apply lanolin mixtures either to the unbranched trunk or to a vigorous 1-year-old lateral shoot. Cut off the terminal portion of the shoot to be treated, usually a segment 10–20 cm. long. Cut off a 4-cm. length of the rubber tubing and half-fill it with the paste containing the compound to be tested. Slip the end of the tubing

containing the paste over the decapitated shoot for a distance of about 1 cm. Double the empty end of tubing over and tie it in this position with a string to protect the lanolin mixture. Repeat this procedure, using different shoots with lanolin alone and also with lanolin plus 3-indolebutyric acid (0.1%), and designate these as a control and a standard treatment.

In an alternate method, use flexible sheet plastic or similar material in place of the rubber tube. Cut strips of the flexible material, 8 × 6 cm.; wrap a strip around the end of each cut branch, and secure it with a rubber band. Melt the lanolin mixture and put about 5 ml. of it into the tube formed by the strip. Slip a short vial over the open end of the tube to protect the lanolin, leaving uncovered the buds immediately below the point of treatment.

Method of taking results—After the lateral shoots on the controls are 15–20 cm. long, determine the average length of the first 3 or 4 lateral shoots nearest the treated portion on each tree. Compare this average with the averages obtained from trees treated with the 3-indolebutyric acid mixture, and with lanolin alone.

Measure with a protractor the angle formed by the trunk of each plant and the first 3 or 4 lateral shoots nearest the treated portion. Compare these measurements with similar measurements on plants given the standard treatment and on control plants.

Suggested standard for comparison—0.1% lanolin mixture of 3-indolebutyric acid.

References

- Preston, A. P., and Barlow, H. W. B. The Use of Growth Substances to Widen Crotch Angles. East Malling Res. Sta. Ann. Rpt. 1950: 76–79. 1951.
Verner, Leif. Effect of a Plant Growth Substance on Crotch Angles in Young Apple Trees. Amer. Soc. Hort. Sci. Proc. 36: 415–422. 1939.
Verner, Leif. Unpublished method. University of Idaho, Moscow.

CELL DIVISION OR MULTIPLICATION

ENDOSPERM METHOD

Based on research by W. T. Jackson, G. P. Pollitt, and H. Sato.

Description of method—Endosperm cells from immature seeds are spread on an agar surface

containing glucose and the desired concentration of the plant growth regulator. Division in these cells following treatment is observed by phase contrast and polarization microscopy. Abnormalities of mitosis, as recorded by means of photography and a rating system, indicate the effect of the test compound on cell division.

Apparatus, Chemicals, and Other Materials

High humidity room (85–90%) at 20°–25° C. for preparation of chambers
 Microscope slides, 50 × 75 mm.
 Cover glasses, 24 × 40 mm., No. 1½
 Razor blades
 Petri dishes
 Flasks
 Alcohol lamp
 Spatulas
 Forceps
 Hot plates
 Filter paper
 Glassware washing facilities
 Analytical balance
 Phase contrast microscope
 Rectified polarizing microscope
 35-mm. camera
 16-mm. time-lapse movie camera with adapters for photomicrography
 (Note: Effective heat absorbers must be inserted between illuminators and microscopes.)
 Monochromatic green light used for illumination of specimen
 Tape recorder to record comments of investigator during period of observation
 1–1,000 p.p.m. of growth-regulating chemical to be tested. (20 p.p.m. of colchicine gives "typical" C-mitosis; 0.1 p.p.m. of 3-chloroisopropyl N-phenylcarbamate is effective)
 Petroleum jelly-lanolin-paraffin sealing compound
 Glucose
 Glass-distilled water

Suggested plant material—Liquid endosperm from a variety of plants (including important crop plants) is suitable. However, the African blood lily (*Haemanthus katherinae* Baker), a member of the Amaryllidaceae family, has thus far proved the most useful.

Preparation and selection of plant material—Mature bulbs of the African blood lily usually flower during the late spring or early summer. The immature fruits are at the proper stage for study during late summer. Select fruits that contain seeds about 6 mm. long. Such seeds possess a liquid endosperm. The cells suspended in the endosperm fluid do not possess a cell wall, will flatten within 30 minutes when placed on an agar surface, and will complete mitosis within 2–4 hr. Each plant, if fruit set is good, will provide nearly 100 fruits suitable for study over a 3-wk. period. Three to 6 years is required to produce a mature bulb from seed or an offshoot, but mature bulbs can be obtained commercially in limited quantities.

Procedure—Select a fruit of proper size, remove the seed, and measure it. If it is 5.5–6.5 mm. long, it will contain several hundred cells in the endosperm. Many of these will be undergoing mitosis. Carry out all operations involved in preparing the material for microscopic examination in a room maintained at 85–90% relative humidity and 20°–25° C. Spread evenly a thin film of liquid 0.5% agar containing 3.5% glucose plus the desired concentration of growth regulator in glass-distilled water on the cover glass. Store this cover glass *immediately* in a moist chamber. Coat one of the microscope slides in the center with a thin layer of the agar solution to match the size of the cover glass. Make a filter paper "gasket" by cutting the center out of a piece of 24 × 40 mm. filter paper leaving a border 1–2 mm. wide. Place the filter paper "gasket" over the coated area of the slide. Wet the filter paper with the agar solution. Store the microscope slide in a moist chamber (petri dish lined with moist filter paper). Cut off the end of the seed. Blot off the first drop of liquid (containing few cells) and gently squeeze the remaining liquid onto the agar on the cover glass. Quickly invert the cover glass and place it on the filter paper "gasket" on the microscope slide. Melt a small amount of 1:1:1 petroleum jelly-lanolin-paraffin sealing compound on a spatula over an alcohol lamp. Working rapidly, apply the melted sealing compound to the edge of the raised cover glass, thus sealing it to the microscope slide. Invert the chamber formed by the slide and cover glass and place it so it is slightly inclined for 15 minutes. The excess liquid drains into the filter paper and the remaining cells flatten to the agar-covered surface of the cover glass due to surface tension. Examine the cells microscopically.

Method of taking results—It is convenient to divide mitosis arbitrarily into 18 stages or events. Select no more than 12 fields of view for study choosing "typical" cells undergoing mitosis. Have an assistant read the stage coordinates, keep track of time for readings, and operate a tape recorder while the investigator gives data verbally concerning the stage of mitosis for each cell. Record by photography or by a rating system, or by both systems, at 10-minute intervals for 4 hr. the progress of mitosis in the cells in each field. Repeat this procedure 5 or 6 times for each concentration.

Make time-lapse 16-mm. movies of individual cells to record detailed effects of the plant growth regulator that may be missed by visual examination or with still-photography techniques. For example, details of chromosome movement can be analyzed with time-lapse photography.

Suggested standard for comparison—Compare behavior of cells during mitosis with behavior of untreated cells and cells treated with 20 p.p.m. of colchicine.

Reference

Jackson, W. T., Pollitt, G. P., and Sato, H. Use of Endosperm of the African Blood Lily (*Haemanthus katherinae* B.) for Studying Mitosis in Living Cells in Both Teaching and Research. (Abstract) Plant Physiol. 39: Sup. lxiii. 1964.

MICROCULTURE METHOD

Based on research by L. E. Jones, A. C. Hildebrandt, A. J. Riker, and J. H. Wu.

Description of method—A microchamber is used to grow cells aseptically in a droplet of medium that contains a measured amount of the test compound. The rate of growth and other characteristics of the treated cells are studied microscopically and compared with those of comparable cells grown in the medium without the test compound. The method is suggested as a means of detecting and measuring substances that influence cell division other than those regulating compounds used as nutrients in this method.

Apparatus, Chemicals, and Other Materials

Prescription bottles, 6 oz.
Reciprocal shaker (60 cycles/minute)
Analytical balance
Microchambers (See "Procedure" for construction.)
Standard microscope slides
Cover slips, 22-mm. square, No. 1
Paraffin oil (U.S.P. heavy paraffin oil)
Honed or flattened teasing needles
Pasteur pipettes
Dissecting microscope
Reagents needed for the preparation of nutrient solution (See "Preparation and selection of plant material.")
500 mg. of indole-3-acetic acid for standard treatment

Suggested plant material—Cells from plant tissue cultures of tobacco (*Nicotiana tabacum* L.), marigold (*Tagetes* spp.), tomato (*Lycopersicon esculentum* Mill.), carrot (*Daucus carota* L.), endive (*Cichorium endivia* L.), lettuce (*Lactuca sativa* L.), potato (*Solanum tuberosum* L.), or bean (*Phaseolus vulgaris* L.) grown with the liquid medium shake culture method. (See "Preparation and selection of plant material.")

Preparation and selection of plant material—To obtain single cells, groups of cells, and nutrient

medium for use in the microchamber, isolate stem callus cells from tobacco or other species. Grow these in 39 ml. of liquid medium contained in the prescription bottles using the reciprocal shaker. Prepare several cultures in this way and place them together in the shaker. Make the medium by dissolving the designated amounts of the following compounds in 850 ml. of water: ¹

800.0 mg. of Na₂SO₄
400.0 mg. of Ca(NO₃)₂ • 4H₂O
180.0 mg. of MgSO₄ • 7H₂O
80.0 mg. of KNO₃
65.0 mg. of KCl
33.0 mg. of NaH₂PO₄ • H₂O
40.0 mg. of Fe₂(C₄H₄O₆)₃
4.5 mg. of MnSO₄ • 4H₂O
6.0 mg. of ZnSO₄ • 7H₂O
0.375 mg. of H₃BO₃
3.0 mg. of KI
3.0 mg. of glycine
0.1 mg. of thiamine
20.0 g. of sucrose
6.0 g. of agar

Dissolve each of the following in the 850 ml. of nutrient solution:

150.0 of coconut milk
2.5 mg. of calcium pantothenate
0.1 mg. of naphthaleneacetic acid
6.0 mg. of 2,4-dichlorophenoxyacetic acid

Procedure—Prepare a microchamber by placing a drop of the paraffin oil near each end of a microscope slide (fig. 8). Lower a cover slip onto each droplet to form risers for a central chamber. Cover the rectangular space on the slide between the 2 risers with the oil. Make certain that the oil also covers the inner edge of each riser. Using a third cover slip, place a droplet of liquid medium (obtained from that employed to produce the original shake-culture if single cells are used) near the center of the cover slip. With a dissecting microscope and teasing needles, remove a small group of cells (2–30) from the shake-culture, or remove cells from the liquid shake-culture with a Pasteur pipette, and place these in the droplet of medium. Invert the cover slip onto the rectangle of oil between the 2 risers in such a manner that the oil surrounds the liquid medium with its enclosed cells and the ends of the top cover slip rest on the inner edge of each riser (fig. 8). Use extreme care to prevent tearing, bending, overheating, or contaminating the cells.

¹ If sunflower tissue is used, for composition of the nutrient medium consult: Hildebrandt, A. C., Riker, A. J., and Duggar, B. M. The Influence of the Composition of the Medium on Growth *in Vitro* of Excised Tobacco and Sunflower Tissue Cultures. Amer. Jour. Bot. 33: 591–597. 1946.

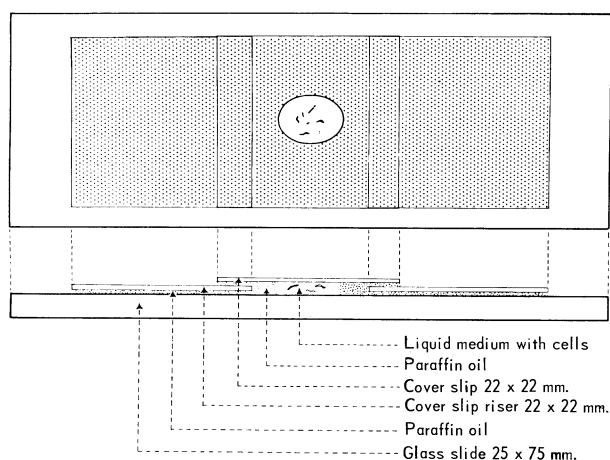


FIGURE 8.—Microchamber for culture of single cells or groups of higher plant cells. (Diagram—courtesy of American Journal of Botany.)

Use the above method to prepare cultures of untreated cells for comparison with cells grown in nutrient containing the test chemical.

Prepare additional microcultures containing the test compound as follows: to obtain a stock solution containing 1 microgram of the test chemical per ml. of culture medium, dissolve completely 250 micrograms of the test chemical in 25 ml. of culture medium. If single cells are involved, use shake-culture medium that has previously supported the growth of cells. If the test compound is relatively insoluble, place the weighed crystals in the medium and pulverize them by crushing them against the bottom of the flask with the flat end of a glass rod. Stir the mixture until the crystals are completely dissolved.

Prepare an exploratory series of dilutions of the test chemical by pipetting 0.2 ml. of the original mixture into a vial containing 0.8 ml. of the culture medium. Mix the diluted compound thoroughly and continue this procedure of dilution until a series of concentrations is obtained, each mixture being one-fifth the concentration of the next highest. Proceed as described above by placing approximately the same number of cells or groups of cells from the shake-culture into a droplet of medium from each of the various levels of dilution. Enclose each droplet in a corresponding microchamber.

Prepare a duplicate series of microcultures containing the various dosages of the test chemical. Place the culture chambers at room temperature or in a controlled temperature chamber at about 25° C. Compare the behavior of the cells in the control nutrient medium with the behavior of cells in medium containing the various amounts of the test compound at week-

ly intervals for about 1 month. (See "Method of taking results" for responses that might occur.)

Use this preliminary series of dosage levels to indicate a more suitable dosage range for studying the effect of the test compound in detail. After determining the appropriate dosage range, repeat the experiment with the proper amounts of the test chemical and record cellular responses as indicated below.

Method of taking results—Observe the microcultures with a microscope using interference, phase, and bright-field illumination. Record the rate of cell division, rate of differentiation (formation of thin-walled elements resembling fibers, formation of scalariform tracheids or giant cells), prevalence of mitochondria, presence or absence of starch or oil droplets, and other detectable changes. Use Janus Green B as a means of differentially staining the mitochondria so they can be identified and their numbers estimated.

Suggested standard for comparison—To compare unknowns, determine the response to known amounts of regulating substances other than those in the nutrient medium, such as indole-3-acetic acid.

Reference

Jones, L. E., Hildebrandt, A. C., Riker, A. J., and Wu, J. H. Growth of Somatic Tobacco Cells in Microculture. *Amer. Jour. Bot.* 47: 468-475. 1960.

ROOT CULTURE METHOD

Based on research by P. R. White; S. M. Caplin.

Description of method—Root tips from tomato seedlings or cuttings cultured *in vitro* are treated by incorporating regulators or other chemicals into the culture medium or into an oil overlay and the effects of these compounds on growth of root cells are determined.

Apparatus, Chemicals, and Other Materials

Scalpel
Forceps
Petri dishes
Filter paper (No. 1 Whatman)
Dark chamber maintained at room temperature of 21°-24° C.
Wide-mouth glass jars
Blotting paper
Heavy, paraffin-coated cardboard
Analytical balance
Bottle, 4 liters
Test tubes

Rubber test tube stoppers
Refrigerator space
Erlenmeyer flasks, 125 ml.
Aluminum foil
Beakers or paper cups, 50 ml.
Autoclave

Salts for nutrient solution:

25 g. of $\text{Ca}(\text{NO}_3)_2$
25 g. of Na_2SO_4
10 g. of KCl
2 g. of NaH_2PO_4
1 g. of MnSO_4
1 g. of ZnSO_4
1 g. of H_3BO_3
1 g. of KI
50 g. of MgSO_4

Vitamins:

0.5–1 g. of glycine
0.5 g. of nicotinic acid
0.5 g. of thiamine
0.5 g. of pyridoxine

Sugar-iron solution:

50 g. of C.P. sucrose
1 g. of $\text{Fe}_2(\text{SO}_4)_3$

Compounds to be evaluated as growth regulators

Sodium hypochlorite solution, such as laundry bleach

Distilled water

Suggested plant material—Ripe tomato fruits, or tomato plants (*Lycopersicon esculentum* Mill.) from which cuttings can be made.

Preparation and selection of plant material—Seed

Method: Select sound, ripe tomato fruits; wash and dry them carefully. *Sterile equipment and aseptic conditions should be utilized throughout the experiment.* With a scalpel, divide the fruit into quarters by making cuts into the fruit deep enough so that it can be broken open easily. Start the incisions about 1 cm. from the stem and cut to the styler end. Break open the fruit and expose the seeds carefully so they do not come in contact with fingers or with the outer, nonsterile surface of the fruit. With sterile forceps, remove 25 mature seeds that have not been touched with the scalpel, and place them in 5 petri dishes (5 seeds per dish) fitted with filter paper moistened with the sterile water. Keep the petri dishes in a dark place at a temperature of about 21°–24° C. for a few days until the seeds germinate and the roots are 2–3 cm. long.

Cutting Method: Grow tomato plants in a greenhouse or field plot. Take 6 straight stem cuttings about 20 cm. long from a healthy tomato plant and remove the leaves. If all these cuttings are from the same plant, genetic variability will be reduced. Wash thoroughly with the 5% hypo-

chlorite disinfectant diluted with 1:10 water. Rinse with sterile water and shake off the surplus. Line the wide-mouth glass jars with sterile blotting paper and add sterile water to a depth of about 3 cm. Cover each jar with a sheet of heavy, paraffin-coated cardboard a little larger than the mouth of the jar. Make a hole in the cardboard and insert the basal end of a tomato cutting through it so that about 15 cm. of stem extends into the moist air above the water surface. Keep the jars containing the cuttings in the dark at a temperature of about 21°–24° C. for about 10 days, or until new roots develop to a length of 3–5 cm.

Procedure—Prepare 4 stock salt solutions, using the best grades of analytical chemicals. Dissolve:

- (1) 15.0 g. $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 4.0 g. KNO_3 , 3.2 g. KCl, 1,000 ml. H_2O .
- (2) 37.5 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10.0 g. Na_2SO_4 , 1,000 ml. H_2O .
- (3) 0.95 g. $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 1,000 ml. H_2O .
- (4) 0.25 g. $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.15 g. $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.075 g. H_3BO_3 , 0.04 g. KI, 1,000 ml. H_2O .

When each stock salt solution is fully dissolved, mix the 4 together slowly. Store this stock solution, which is 10 times the concentration needed, in the dark or in a black bottle.

Prepare a stock solution of vitamins by dissolving 300 mg. of glycine, 50 mg. of nicotinic acid, 10 mg. of thiamine, and 10 mg. of pyridoxine in 100 ml. of water. This solution is 100 times the concentration needed. Pour 20-ml. aliquots of this vitamin stock solution into test tubes, stopper the tubes, and store them in a refrigerator.

Sucrose is used as a carbon source for the tissue cultures. Dissolve 40 g. of c. p. sucrose in 1 liter of water. Dissolve 10 mg. of $\text{Fe}_2(\text{SO}_4)_3$ in 100 ml. of water, discard half the quantity, and add the remainder to the sugar solution. (Diluting and discarding half the ferric sulfate solution eliminates the necessity of weighing accurately a quantity of the salt less than 10 mg.)

Mix the sugar-ferric sulfate solution with 200 ml. of the stock salt solution and 20 ml. of the vitamin stock solution, and add sufficient water to make 1,600 ml. Distribute 40 ml. of this complete nutrient solution to each of the desired number of the 125-ml. Erlenmeyer flasks. Cover with squares of foil, flaming the outside to insure sterility. Cap with a 50-ml. beaker or small paper cup to keep out dust while allowing air exchange. After being autoclaved (15 lb. pressure per sq. in. for 20 minutes) and allowed to cool, the nutrient in each flask is ready for the addition of 10 ml. of sterile distilled water con-

taining the test compound at 5 times the final concentration desired. For control flasks, add 10 ml. of the sterile water.

Select 10 roots, 2–3 cm. long, from the germinated seeds; sever them with a sharp scalpel and transfer each root to a flask of nutrient. To obtain a sufficient number of root tips from a single source, culture these roots for a week; then with scissors cut 1-cm. tips from the main root and well-established branch roots. With a sterile bacteriological loop, insert each root tip into a culture flask containing fresh nutrient. If rooted cuttings are used, cut 1-cm. root tips and insert them into culture flasks in the same manner. To reduce the possibility of genetic effects influencing the results, use root tips derived originally from a single seed or from cuttings from a single plant. Or, if a larger amount of material is needed for a more extensive experiment, label and distribute such genetically uniform roots so that one or more are used for each treatment.

Treat the excised roots by adding plant regulators in the required amounts aseptically (i.e., using sterile solutions and utensils) to the nutrients as described above. A range of final concentrations from 10^{-13} to 10^{-1} g. per liter, prepared by serial dilution, is suggested. Do not autoclave the aqueous solutions of regulating chemicals. Some regulating chemicals are relatively insoluble in water in the acid, ester, or amide form. Employ a salt form of these compounds to facilitate their use in this method. Use a group of untreated solutions, each brought to a volume of 50 ml. with sterile distilled water, as controls. Place the culture flasks in the dark at 21°–24° C.

Method of taking results—Since roots grown in culture do not undergo secondary thickening, and therefore the diameter remains constant to within a few millimeters of the growing point, length measurements are an accurate measure of volume. Without removing the root from the culture flask, make day-by-day measurements from the outside using a flexible rule, either by bending the rule to approximate the root curvature or by washing the root up onto the wall of the flask so that it lies straight. Also measure growth at the end of the week by removing the root tissue and obtaining wet or dry weights.

Variation used by Caplin—For prolonging the time between transfers of tissues grown on culture, S. M. Caplin suggests that tissue explants of uniform size of carrot secondary phloem can be grown on agar culture medium containing White's basal nutrients plus coconut milk (or other growth-promoting substances) and over-

layered with mineral oil. The resultant slower growth of the cultures under mineral oil prolongs the subculture period from the usual 4–6 wk. to 4–6 mo., with attendant savings in effort, time, and materials. The fact that plant tissue cultures grow under mineral oil suggests a method to test the effectiveness of growth-regulating substances that are relatively insoluble in water, for example, esters of growth regulators. Also suggested is the possibility of uniform lateral application to a culture tissue of a drop of oil containing the test material.

Suggested standard for comparison—Roots grown in a control solution to which only the equivalent amount of sterile, distilled water has been added serve as a standard of comparison.

References

- White, P. R. *The Cultivation of Animal and Plant Cells*. Ed. 2 (rev.), 228 pp. Ronald Press Co., New York. 1963.
Caplin, S. M. Mineral Oil Overlay for Conservation of Plant Tissue Cultures. *Amer. Jour. Bot.* 46: 324–329. 1959.
Caplin, S. M. Effect of Initial Size on Growth of Plant Tissue Cultures. *Amer. Jour. Bot.* 50: 91–94. 1963.

SINGLE ISOLATED CELL METHOD

Based on research by A. C. Hildebrandt; W. H. Muir, A. C. Hildebrandt, and A. J. Riker.

Description of method—Single cells obtained from tissue of marigold or tobacco grown in liquid culture are placed on filter paper sheets that have been resting on tissue culture hosts. After several weeks, uniform young cultures of tissue that develop from the single cells are transferred to agar medium containing the compound to be tested. Comparable cultures are transferred to agar without the compounds added and these serve as controls. Responses are detected by microscopic examination of the cultures at an early stage of their development or later by microscopic examination. Growth of the cultures on the medium containing the test compound is compared with that of cultures on the control medium. Acceleration or suppression of growth or differentiation of cells compared with that of the control cultures is a measure of the growth-regulating properties of the compound tested.

Apparatus, Chemicals, and Other Materials

Analytical balance
Scalpel
Reciprocal shaker
Dissecting microscope

Micromanipulator or "biscuit cutter" type isolator (See "Microisolation device," p. 113.)

Flattened needle

Forceps

Sterile filter paper, Reeve Angel, crepe surface, No. 202, 8×8 mm.

Petri dishes

Mm. rule

Liquid culture medium

Agar medium (See "Stem tissue culture method," p. 21.)

Suggested plant material—Tissue cultures from crown galls of marigold (*Tagetes erecta* L. var. Sunset Giant) of crown gall origin or from the normal stem of tobacco (*Nicotiana tabacum* L. var. Havana 38).

Preparation and selection of plant material—Prepare tissue cultures from crown gall of marigold or from the normal stem tissue of tobacco. Grow these cells in liquid culture on a reciprocal shaker. (See p. 15.)

Procedure—Remove suspensions of cells from the liquid culture and disperse them over a firm agar medium in a petri dish. Locate single cells under a dissecting microscope and use the "biscuit cutter" type isolator or micromanipulator to remove them individually with a flattened needle under aseptic conditions. Place each cell on the square sheet of sterile filter paper that has been resting for 2 or more days on the top surface of a young tissue culture (about 7 mm. in diameter) of marigold or tobacco growing on an agar medium.

Return each filter paper square to the upper surface of the "host" culture. These operations require speed to avoid exposing the single-cell isolates to excessive light and desiccation. The upper surface of the filter paper should not come into direct contact with the underlying host tissue. Either marigold or sunflower tissue cultures may be used as hosts for single marigold cells. Use tobacco tissue cultures as hosts for single tobacco cells.

As the host culture becomes senescent, the young culture it supports will also lose vigor. The filter paper with its growing tissue must, therefore, be transferred to a fresh host one or more times during the test to maintain an optimum rate of growth.

As a matter of convenience, one single-cell culture has been referred to in describing this method. Actually, many of these cultures are necessary in order to determine the growth-regulating properties of a chemical by this means. The number of replications needed and the total cultures required must be determined

for each experiment involved. Stocks of single-cell origin may be carried through 4 or more agar medium transfers, the period between each transfer being approximately 5 wk. The cultures from single cells may be subdivided into portions approximately 4 mm. in diameter without diminution in growth rate as a result of this division. In this way a sufficient number of replicate cultures can be obtained, all of which came from the same single cell.

To test the compound, transfer the cultures resulting from a single cell directly to agar medium that contains appropriate amounts of the regulator to be tested.

Method of taking results—Acceleration or suppression of growth of the tissue developed from a single cell, compared with that of comparable cultures on untreated agar, indicates response to the chemical used. Differentiation of cells of cultures supported by agar medium containing the test chemical also indicates response to the compound under test. This response is detected by macroscopic examination or by examination of the culture through a dissecting microscope.

Suggested standard for comparison—No standard treatment is suggested.

References

- Hildebrandt, A. C. Stimulation or Inhibition of Virus-Infected and Insect-Gall Tissues and Single-Cell Clones. *Nat. Acad. Sci. Proc.* 44: 354-363. 1958.
Muir, W. H., Hildebrandt, A. C., and Riker, A. J. Plant Tissue Cultures Produced from Single Isolated Cells. *Science* 119: 877-878. 1954.

SOYBEAN CALLUS TISSUE METHOD

Based on research by C. O. Miller.

Description of method—Soybean callus tissue previously grown on a nutrient medium containing kinetin is transferred to a medium without kinetin. Compounds or extracts from plant tissues to be tested for kinetin-like properties are added and the resultant increase in cell division is used as a measure of the activity of the compounds or extracts tested.

Apparatus, Chemicals, and Other Materials

Erlenmeyer flasks, 125 ml.

Razor blades

Space illuminated by fluorescent light (25 foot-candles) and maintained at 27° C.

Autoclave

Mercuric chloride

Kinetin

Basal medium for soybean assay:

Component	Concentration mg./liter
Ca(NO ₃) ₂ -----	347.0
KNO ₃ -----	1,000.0
NH ₄ NO ₃ -----	1,000.0
KH ₂ PO ₄ -----	300.0
MgSO ₄ -----	35.0
KCl -----	65.0
Sodium ferric ethylenediamine- tetraacetate -----	32.0
MnSO ₄ -----	4.4
ZnSO ₄ -----	1.5
H ₃ BO ₃ -----	1.6
KI -----	0.8
Glycine -----	2.0
Nicotinic acid -----	0.5
Thiamin • HCl -----	0.1
Pyridoxine • HCl -----	0.1
Sucrose -----	30,000.0
Agar -----	10,000.0
Adjust pH to 5.8 with NaOH solution	

Suggested plant material—Seeds of soybean (*Glycine max* (L.) Merrill variety Acme).

Preparation and selection of plant material—Sterilize soybean seeds by soaking them in 0.1% mercuric chloride for 15 minutes. Rinse the seeds 4 times with sterile distilled water. Plant 3 seeds in each of a number of Erlenmeyer flasks containing 50 ml. of the medium described above.

After the seeds have germinated, remove the cotyledons and cut them into blocks 4 × 4 × 2 mm. Place 1 block per flask on the medium shown above to which 0.5 mg./liter of kinetin has been added. After about 3 wk., subculture the wound callus, placing it on the same kind of medium. Continue to subculture the tissue until a sufficient quantity of stock culture has been accumulated to initiate tests.

Procedure—Using stock tissue cultures that have turned slightly yellow while being grown on nutrient medium containing kinetin, place 3 small blocks of this tissue (approximately 2 × 2 × 2 mm.) on 50 ml. of the medium without the kinetin contained in a flask. Use 4 such plantings for each treatment and retain 4 of these flasks as controls. Prepare other cultures of tissue in the same way, but add to the nutrient the compound or extracts to be tested. Keep the flasks under fluorescent light of about 25 foot-candles and at a temperature of 27° C.

Method of taking results—After 4 or 5 days, examine the cultures to determine in a preliminary way which of the compounds being tested accelerated cell division, as evidenced by a whiter and larger appearance of the tissue

pieces. Weigh the tissue pieces at the end of 2 wk., or at the end of a longer period if larger weight differences are desired.

Response in terms of fresh weight is directly proportional to the logarithm of the kinetin concentration over a range of about 0.004–10 mg./liter.

Suggested standard for comparison—Kinetin at various concentrations added to the nutrient described above. Range of concentrations 1 through 1,000 × 10⁻⁸ M.

Reference

Miller, C. O. Kinetin and Kinetin-Like Compounds. *Mod. Methods of Plant Anal.* 6: 194–202. 1963.

STEM TISSUE CULTURE METHOD

Based on research by A. C. Hildebrandt and A. J. Riker; B. E. Struckmeyer, A. C. Hildebrandt, and A. J. Riker; P. R. White.

Description of method—Squares of phloem tissue from willow are grown on nutrient agar containing regulating chemicals and effects of the chemicals on growth and tissue development determined.

Apparatus, Chemicals, and Other Materials

Sharp knife
Scalpel
Tweezers
Cork or soft pine board
Bottle, 5 liters
Erlenmeyer flasks, 125 ml.
Test tubes
Rubber test tube stoppers
Refrigerator space
Cotton
Gauze
Beakers or paper cups, 50 ml.
Autoclave
Analytical balance
95% alcohol
Chemicals for complete nutrient solution (See p. 17.)
Compounds to be evaluated as growth regulators
Agar (Difco "Noble" brand)
Distilled water

Suggested plant material—Young willow tree (*Salix nigra* L. or *S. caprea* L.).

Preparation and selection of plant material—Cut from the young willow tree a piece of branch or stem about 30 cm. long and 3–10 mm. in diam-

eter. Surface-sterilize by first scrubbing the surface with soap and water. Dip the branch into 95% alcohol several times and burn the alcohol off each time after the branch is dipped.

Procedure—Prepare a standard salt solution and a vitamin stock salt solution, as described on page 21, but use only half the volume of water. Use sterile water redistilled in Pyrex for all solutions. Dissolve 40 g. of c.p. sucrose in 400 ml. of water. Dissolve 10 mg. of $\text{Fe}_2(\text{SO}_4)_3$ in 100 ml. of water, discard half the quantity, and add the other half to the sugar solution. Prepare a 1% agar solution by dissolving 10 g. of Difco "Noble" agar (or its equivalent in thoroughly leached agar) in 1 liter of hot water. Combine the agar, the sugar-ferric sulfate solution, 100 ml. of stock salt solution, and 10 ml. of vitamin stock solution, and add sufficient water to make 1,600 ml. Mix together thoroughly and divide the hot nutrient-agar solution into aliquots, each containing 60 ml., and autoclave. To a 60-ml. aliquot of the hot agar solution, add 15 ml. of warm aqueous solution of the test compound. This solution should be prepared with sterile distilled water in sterile utensils and should be 5 times the desired final concentration. Use salt forms of the growth regulator. While the agar-chemical mixture is still hot, distribute equal volumes of it among 5 sterile test tubes. Repeat, using other concentrations and regulators, and prepare one set, using distilled water in place of the regulator, as controls. A range of concentrations of the regulator from 10^{-13} – 10^{-1} g. per liter is suggested. Plug the test tubes with cotton, and cap them with 50-ml. beakers or paper cups to keep out dust. If the nutrient-agar medium does not solidify satisfactorily on cooling, adjust the acidity to a pH not lower than 5.5 with 0.1 N sodium hydroxide.

Lay the sterile branch on a sterile block and, with a sterile scalpel, make a series of transverse cuts through the branch, about 1 mm. apart, to obtain 10 or 15 branch sections or disks. If the branch is 5 mm. or more in diameter, cut the disks into several sectors. If a smaller branch is used, merely cut the disks in half. Cut off and discard outer cortical tissue (bark) and much of the wood. The remaining blocks should be rectangular with the long axes tangential to the original surface and transversed by the cambium.

After the nutrient agar has cooled and solidified, transfer one of the prepared blocks to each tube, using sterile tweezers. Push the blocks part way into the agar so they are left half exposed and half submerged. Stopper the tubes with cotton and set them aside in the dark at a room temperature of 21° – 24°C .

Method of taking results—After 4–6 weeks, compare the growth of tissue on nutrient agar containing various concentrations of growth regulators with growth on nutrient agar alone. Proliferation should occur at or just above the agar level, the new growth spreading out over the agar in the control tubes. Study the effects of the growth regulators on development of the treated tissue during a 4- to 6-week observation period. After this period, remove the samples of tissue grown on nutrient agar and on nutrient agar plus regulating chemical, and compare their wet or dry weights. In addition, record anatomical effects, such as total number of cells, number of tracheal elements per unit area, and gross morphological effects.

Suggested standard for comparison—No standard suggested.

References

- Hildebrandt, A. C., and Riker, A. J. Influence of Some Growth-Regulating Substances on Sunflower and Tobacco Tissue *in vitro*. Amer. Jour. Bot. 34: 421–427. 1947.
 Struckmeyer, B. E., Hildebrandt, A. C., and Riker, A. J. Historical Effects of Growth-Regulating Substances on Sunflower Tissue of Crown-Gall Origin Growth *in vitro*. Amer. Jour. Bot. 36: 491–495. 1949.
 White, P. R. The Cultivation of Animal and Plant Cells. 239 pp. Ronald Press Co., New York. 1954.

SUBMERGED CULTIVATION METHOD

Based on research by L. G. Nickell.

Description of method—A culture is maintained in the form of single plant cells, small clumps of cells, or both, submerged in a liquid medium. Growth measurement is determined by first measuring the number or volume of cells in an aliquot of culture medium immediately after inoculating the medium with a cell suspension and then measuring the number or volume of cells again at the end of the test. The number or volume increase of cells in medium containing the chemical to be tested is compared with that which occurs in the control culture medium.

Apparatus, Chemicals, and Other Materials

Analytical balance
 Erlenmeyer flasks, 300-ml.
 Scalpel
 Autoclave
 Rotary shaker, 230 r.p.m.
 Pipettes, 1 and 10 ml.
 Inoculating loop
 Centrifuge
 Graduated centrifuge tubes, Kolmer type or equivalent

Hemocytometer counting chamber

White's medium (See p. 17.) with addition of:

2,4-dichlorophenoxyacetic acid (0.6 p.p.m.)

Coconut milk (18% of final volume) or mixture of yeast and malt extracts (0.1% each)

Agar

500 mg. of 2,4-dichlorophenoxyacetic acid, gibberellic acid A₃, or indole-3-acetic acid for standard treatment

Suggested plant material—Pole bean (*Phaseolus vulgaris* L.); century plant (*Agave toumeyana* Trel.); rose (*Rosa* spp.) Paul's Scarlet variety; holly (*Ilex aquifolium* L.); sugarcane (*Saccharum officinale*).

Preparation and selection of plant material

Sterilize several seeds of the plant desired, place them on a nutrient medium (1% agar added to nutrient described under "Root culture method," p. 17) in flasks and allow the seeds to germinate and develop into plants. Aseptically excise leaves, stems, roots, and other organs and place them again on the agar medium to induce callus formation. Continue this type of culture until a sufficient number of cultures are obtained. When the callus is 0.5–1 cm. in diameter, make subcultures.

Procedure—After making several subcultures, transfer tissue masses to White's medium without agar. To do this, dispense 50 ml. of the liquid medium into the flasks and autoclave 15 minutes at about 15 lb./sq. in. pressure before the transfer is made. Then transfer tissue masses from agar cultures to this sterile medium after it has cooled. Shake flasks on rotary shaker at 230 r.p.m. or use such methods as unidirectional shaking, propeller stirring, or alternating rotary shaking with baffles. Continuous shaking of the culture for several days reduces the culture tissue mass to a submerged living cell suspension.

Method of taking results—Determine the number of cells per unit volume by microscopic examination immediately after initiation of the test and compare with that in an equal volume obtained at the end of the culture period before the next subculture. The increase in number of cells indicates rate of growth.

Also determine pack-cell volume by centrifuging cells contained in a 5-ml. portion of the culture at the beginning and end of growth periods. Centrifuge the 5-ml. cell suspensions in the Kolmer-type centrifuge tubes or equivalent at 1,500 r.p.m. for 2–3 minutes until cells are tightly compacted. Read the volume of cells directly from the calibrated centrifuge tube.

An alternative method of determining increase in cell numbers, which also yields data concerning changes in the structure of cells, is microscopic examination. Take the required volume of the suspension for a hemacytometer counting chamber and then make counts as directed. At the same time, compare the structure of cells grown in medium containing the test compound with the structure of those grown in the nutrient medium alone.

Suggested standard for comparison—0.6 p.p.m. of 2,4-dichlorophenoxyacetic acid compared with absence of the acid or presence of gibberellic acid, indole-3-acetic acid, or other growth regulators.

References

- Nickell, L. G. The Continuous Submerged Cultivation of Plant Tissue as Single Cells. *Natl. Acad. Sci. Proc.* 42: 848–850. 1956.
 Nickell, L. G. Growth Effects of Antibiotics on Plant Cells and Their Reaction to a Modified Gram Stain. *Phyton* 11: 93–96. 1958.
 Nickell, L. G., and Tulecke, W. Submerged Growth of Cells of Higher Plants. *Jour. Biochem. and Microbiol., Technol. and Engn.* 2: 287–297. 1960.
 Nickell, L. G. Submerged Growth of Plant Cells. *Adv. Appl. Microbiol.* 4: 213–236. 1962.
 Nickell, L. G. Tissue and Cell Cultures of Sugarcane—Another Research Tool. *Hawaiian Planters' Rec.* 57: 223–229. 1964.

TOBACCO PITH METHOD (MICRO)

Based on research by W. Bottomley, N. P. Kefford, J. A. Zwar, and P. L. Goldacre.

Description of method—Blocks of tobacco pith are placed aseptically on a basal nutrient solution containing indole-3-acetic acid. Addition of kinetin or plant extracts with kinetin-like substances induces the formation of neoplasm on the pith blocks. Increase in the number of these neoplastic cells is an indication of the amount of kinetin or kinetin-like substance present in the test material.

Apparatus, Chemicals, and Other Materials

Petri dishes
 Scalpel
 Test tubes, 7.5 × 1.2 cm., fitted with aluminum caps
 Filter paper strips, Whatman No. 1, 4 × 0.9 cm.
 Autoclave
 Dark chamber maintained at a high humidity and 25° C.
 Dissecting microscope, 10× magnification
 Distilled water
 Sodium hypochlorite solution
 250 mg. kinetin for standard of comparison

Make a basal stock nutrient solution as follows and adjust it to pH 5.8:

0.3 g. of KH_2PO_4
 1.0 g. of KNO_3
 1.0 g. of NH_4NO_3
 0.5 g. of $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$
 0.035 g. of MgSO_4
 0.065 g. of KCl
 0.8 mg. of KI
 4.4 mg. of MnSO_4
 1.5 mg. of ZnSO_4
 1.6 mg. of H_3BO_3
 0.1 mg. of Thiamin $\cdot \text{CHl}$
 0.5 mg. of Nicotinic acid
 0.1 mg. of Pyridoxine $\cdot \text{HCl}$
 1.75 mg. of Indole-3-acetic acid
 20.0 g. of Sucrose

Add sufficient distilled water to make 1 liter of solution.

Suggested plant material—Stem pith tissue of tobacco (*Nicotiana tabacum* L.) cultivar Wisconsin Havana 38.

Selection and preparation of plant material—Grow the tobacco plants in soil under greenhouse conditions using approximately 30° C. day temperature and 25° C. night temperature. Use high mineral nutrition and subject the plants to natural light of high intensity to produce thick, easily worked stems.

Cut pieces of stem 15 cm. long and sterilize them by soaking them in sodium hypochlorite solution for 30 minutes or by aseptically peeling off the outer layers of tissue. Cut the stem pieces transversely into cylinders 10 mm. long. With longitudinal cuts, make blocks about $10 \times 5 \times 3$ mm., taking care to avoid including any vascular tissue. Store the blocks in petri dishes containing a small amount of the sterile basal medium before transferring the blocks to test tubes.

Procedure—Prepare a dilution series by placing the substance to be tested in the basal medium and using this medium as a diluent. Place a filter paper strip in one of the test tubes. Add 1.0 ml. of one of the prepared solutions to the test tube. Close the test tube and prepare additional test tubes with paper and solution to give 3 replicates for each dilution. Sterilize the tubes at a pressure of 18 lb./sq. in. for 5 minutes. Transfer aseptically one pith block to each tube so the block contacts the medium only through the filter paper. Close the tubes and place them in a rack so that they lie at an angle of about 20° from horizontal. Place all cultures in darkness at high humidity and at 25° C.

Method of taking results—Inspect the cultures in light at the end of 14 and 21 days using the dissecting microscope. Cytokinin activity can be estimated by assessing the area of the block that is covered with neoplasm. This varies from no cells, through a few small clusters of tiny cells, to the stage where the whole block is covered with new cells. The new cells are readily recognized, since they are smaller than the cells of the original pith tissue, round, and opaque. If a dilution series is used, an index of cytokinin activity can be obtained by taking the reciprocal of the lowest concentration that causes neoplasm to form.

Suggested standard for comparison—Response can be compared with the increase in cell division induced when a dilution series of kinetin is added to the basal medium.

Reference

Bottomley, W., Kefford, N. P., Zwar, J. A., and Goldacre, P. L. Kinetin Activity from Plant Extracts. I. Biological Assay and Sources of Activity. *Austral. Jour. of Biol. Sci.* 16: 395–406, 1963.

CELL ELONGATION

The first test of this kind to be accepted for wide use in phytohormone research was the *Avena* coleoptile curvature test. This has been described in great detail by F. W. Went and K. V. Thimann, *Phytohormones*, Macmillan Co., New York, 1937, and, for this reason, is not described here.

OAT COLEOPTILE SECTION METHOD

Based on research by D. H. McRae and James Bonner; Pär Fransson; J. C. Sirois.

Description of method—Segments² of oat coleoptiles of uniform length are incubated in solutions of the test compounds. A comparison of length measurements of the segments maintained in the test solutions with those of comparable segments maintained in a standard solution indicates the extent to which the growth rate is affected by each compound.

² The term "segment" is generally used in this compilation instead of "section" since the term "section" often refers to a thin slice viewed with a microscope.

Apparatus, Chemicals, and Other Materials

Stainless steel, glass, plastic, or enamel-coated pans about 35 × 25 × 5 cm.

Standard Avena room (or growth chamber) maintained at 25°–26° C. and 90% relative humidity. (If a growth chamber is used instead of a standard Avena room, the chamber should be conveniently near a dark room where manipulations can be made.)

Red bulb, 10 w.

Analytical balance

Centimeter rule graduated to 0.1 mm. or a calibrated ocular micrometer to use with dissecting microscope

Dissecting microscope

Microscope slides

Forceps

Double-bladed cutting tool constructed of razor blades held parallel to each other exactly 5 mm. apart (See "Segment cutters," p. 101.)

Petri dishes

1% sodium hypochlorite solution

0.0025 M potassium maleate buffer solution containing 3% sucrose prepared with water distilled in Pyrex

0.01–200 mg. of each compound to be tested

175 mg. of indole-3-acetic acid for standard treatment

Detergent

Distilled water

Vermiculite or perlite

Suggested plant material—Seeds of oat (*Avena sativa* L.) Siegeshafer or Victory variety. Seeds of the victory variety can be obtained from the Department of Genetics, University of Alberta, Edmonton, Alberta, Canada, as No. C.A.N. 426, or from the Plant Products Division, Production and Marketing Branch, Canada Department of Agriculture, Ottawa, Ontario, as Victory oats.

Preparation and selection of plant material

Sort the seeds eliminating any that are shriveled or diseased. Sterilize the selected seeds for 10 minutes in the 1% sodium hypochlorite solution containing a small amount of detergent, and then soak the sterilized seeds in distilled water for 1 hr. Fill the pans with vermiculite (or perlite) to a depth of approximately 4 cm. Wet the vermiculite thoroughly with distilled water and allow it to equilibrate overnight. Pour off excess water from one corner of the pan. Remove about 0.6 cm. of vermiculite. Firm and smooth the remaining vermiculite but do not pack it; then sow the seeds on the surface of this prepared bed. Cover with the previously removed vermiculite; smooth and firm it.

Fransson suggested a variation of this method of obtaining plant material that is more time consuming but results in a higher number of coleoptiles with straight growth. Plant the soaked seeds in vermiculite with their husks removed, spacing each seed about 1 cm. apart. Insert the seeds into the vermiculite with the embryo ends downward to such a depth that the upper ends of the seeds are 0.5 cm. below the surface. Cover the entire seedbed with a thin layer of vermiculite. Place the pans in the growth chambers at about 25° C. and allow the seeds to germinate under continuing low-intensity red light (10-w. red bulb 1–2 m. from trays).

Eighty-two to 84 hr. after sowing the seeds, select for use in the tests plants of uniform length (2.8–3.3 cm.) with uniformly straight coleoptiles. This and the further operations may be carried out in the growth room under phototropically inactive orange (longer than 550 millimicrons) light. Corning Filters 243 or 348 are satisfactory. Sprinkle distilled water over the seedlings approximately 24 hr. later, and again about 12 hr. before harvesting. This facilitates the handling of the coleoptiles and increases the sensitivity of the isolated segments to added auxins, particularly to indole-3-acetic acid.

Procedure—Excise with the double-bladed cutting tool one 5-mm. segment from each selected coleoptile. The upper cut should be made no closer than 2–3 mm. from the coleoptile tip and all segments should be cut at a uniform distance from the tip. The piece of leaf contained within the coleoptile segment may be allowed to remain in place. Immediately after cutting the segments, place them in groups of 20 for about 1 hr. in a portion of basal medium consisting of water distilled in Pyrex, 3% sucrose, and 0.0025 M potassium maleate buffer (pH 4.5). This will bring all segments into equilibrium and wash out the endogenous auxin from the segments. In this way limited growth of the controls is obtained. After this pretreatment period, place one set of 20 segments in the fresh basal medium to serve as controls. Place another set of 20 in fresh basal medium to which the test compound has been added and the final mixture adjusted to pH 4.5. Suggested range of concentrations to be tested is 10^{-8} – 10^{-3} M solutions. Prepare as many replicates of 20-segment sets as needed (usually a total of 2 or 3 sets). Incubate the segments in the solutions in the dark for 12 hr. at 25°–26° C.

Method of taking results—At the end of the incubation period, transfer the segments (20 at a test) to a microscope slide, placing them side

by side, parallel, in a row. Add 1 or 2 drops of the solution in which they have been incubated to keep them from drying out. Transfer the slide to the stage of the microscope and measure and record the length of each segment to the nearest 0.1 mm., using a graduated cm. rule or ocular micrometer. Compare the final length of the segments incubated in the basal medium plus test compound with the length of those incubated in basal medium alone. Of particular value is information concerning the concentration of test compound that elicits one-half the maximal growth effect and the growth effect elicited by the test compound when applied in optimal concentrations.

Suggested standard for comparison—Basic solution containing 0.175 p.p.m. (10^{-6} M) of indole-3-acetic acid.

Sirois suggested a modification of the Coleoptile Section Method that makes possible direct reading of auxin concentrations and the use of two segments from each coleoptile, thus doubling the number of assays hitherto possible with a given number of coleoptiles. The range, accuracy, and sensitivity of the modified assay are comparable to those of the standard *Avena* curvature test.

Surface sterilize oat seeds of the Brighton variety with 2% sodium hypochlorite solution. Rinse the seeds in tapwater for 1 hr. and soak them in distilled water for 2 hr. Germinate the seeds on tissue paper in distilled water under red light for 20 hr. at 24° C. and in darkness for 48 hr. at 26° C. Select coleoptiles 20 mm. \pm 2 mm. long and decapitate them 3 mm. from the apex. (See "Segment cutters," p. 101.) Excise two 5-mm. segments from each coleoptile and place each in distilled water contained in separate petri dishes for 3 hr.

Place a 2.5-cm.² piece of chromatographic paper in a petri dish and add 2 ml. of 0.005 M K_2HPO_4 –0.0025 M citric acid buffer at pH 4.8. Place 10 coleoptile segments on the paper. Prepare as many dishes in this manner as required, adding to some the desired amounts of indole-3-acetic acid or test compound. Incubate the dishes containing the coleoptiles in darkness at 26° C. for 20 hr. and then measure the length of the segments under 10 \times magnification. Elongation of the coleoptile segments is directly proportional to the concentration rather than to the logarithm of the concentration of indole-3-acetic acid.

References

- Modification suggested by Pär Fransson, Institute of Plant Physiology, Lund, Sweden.
McRae, D. H. and Bonner, James. Chemical Structure and Antiauxin Activity. *Physiol. Plant.* 6: 485–510. 1953.

Sirois, J. C. Studies on Growth Regulators. I. Improved *Avena* Coleoptile Elongation Test for Auxin. *Plant Physiol.* 41: 1308–1312. 1966. [Plant Research Institute, Research Branch, Canada Department of Agriculture, Ottawa, Ontario.]

BEAN HYPOCOTYL METHOD

Based on research by R. L. Weintraub, J. W. Brown, J. A. Throne, and J. N. Yeatman.

Description of method—A single droplet containing a known amount of a growth regulator is placed on one side of a decapitated bean hypocotyl and after 5 hours the degree of curvature is measured and compared with curvature induced by a standard compound such as 2,4-dichlorophenoxyacetic acid.

Apparatus, Chemicals, and Other Materials

Vermiculite, No. 3
Galvanized iron pans, 51 cm. wide \times 66 cm. long \times 8 cm. deep perforated for drainage
Light-tight room at 31° C. with relative humidity of $60 \pm 10\%$
Red fluorescent lamps, 40 w.
Sharp knife or razor blade
Board 2 \times 2 \times 43 cm. with row of V-shaped notches along one edge
Tuberculin-type glass syringe, 0.25 ml.
No. 27 stainless-steel hypodermic needle with end cut off at right angle
Micrometer caliper with anvil removed and frame modified to hold syringe securely or dial-type micrometer modified to hold syringe, syringe-micrometer assembly calibrated to 0.0001-ml. units with mercury
60-w. Mazda lamp with Wratten OA filter
Pan 10 cm. wide \times 41 cm. long \times 5 cm. deep
Bromide photographic paper
Movable-arm protractor
Analytical balance
Chemicals to be evaluated for cell-elongating activity
Relatively pure 2,4-dichlorophenoxyacetic acid for standard treatment
95% ethanol
Photographic developer
Petroleum jelly

Suggested plant material—Young bean plants (*Phaseolus vulgaris* L.)—Black Valentine, Pinto, Red Kidney, or other varieties.

Preparation and selection of plant material—Germinate the seeds and grow the seedlings in pans containing vermiculite until the plants are uniformly 8–13 cm. tall. This requires about 4 days, and is done in a light-tight room

at 31° C. with relative humidity of $60 \pm 10\%$. Suspend red fluorescent lamps (40-w.) 1–2 m. above the developing plants to induce straight stem growth.

Remove plants from vermiculite carefully to prevent serious root damage. Select plants of uniform size and, with a sharp knife, remove tops at the base of the hypocotyl crook. Arrange 20 decapitated plants so that a portion of the hypocotyl just above the roots is in a notch of the board support. Place a dab of petroleum jelly over each portion to hold it in place. Rest the ends of the board on the edges of the shallow pan so the roots are submerged in tapwater for 2 hr. before the test.

Procedure—Dissolve the chemical to be tested for cell-elongating activity in 95% ethanol. This solution should contain 0.065 μg . of the chemical per 0.0015 ml. of alcohol if comparison is to be made with the cell-elongating effect of 2,4-dichlorophenoxyacetic acid. Load the syringe with the growth regulator-ethanol solution and apply a single 0.0015-ml. droplet to one side of each bean stem 5 mm. below the cut surface. Treat each of the 20 stems per board; then repeat the operation for each dosage level of each compound to be tested. Always apply the treatment in the same relative position on each stem. Leave one lot of stems untreated and treat another with the solvent only (95% ethanol) for comparison. Use a 60-watt Mazda lamp fitted with a Wratten OA filter to furnish light during application of treatments and for a 5-hr. test period thereafter.

Method of taking results—Five hours after treatment, excise the 20 hypocotyl stumps just above the support and lay them on a sheet of glass over a piece of bromide paper. Orient the stumps so that the treated side is always on the left. Briefly expose them to a white light from a source directly above to produce a shadowgraph. Develop the bromide paper and measure the angle of curvature of the hypocotyl stumps with the protractor. The average degree of curvature of treated stumps in comparison with that of the untreated stumps indicates the relative cell-elongating property of the chemical.

Suggested standard for comparison—2,4-dichlorophenoxyacetic acid applied in 95% ethanol so that each 0.0015-ml. droplet contains 0.065 μg . of the acid.

Reference

Weintraub, R. L., Brown, J. W., Throne, J. A., and Yeatman, J. N. A Method for Measuring Cell-Elongation-Promoting Activity of Plant-Growth Regulators. *Amer. Jour. Bot.* 38: 435–440. 1951.

BEAN SECOND INTERNODE METHOD

Based on research by J. W. Mitchell and Greta D. York.

Description of method—In this micro method, the compound or extract to be tested is uniformly dispersed in 250 micrograms of fractionated lanolin. The mixture is applied with the aid of a dissecting microscope to the second internode of a bean stem when the internode is about 0.7–1.0 mm. long. Within limits, elongation of the internode is proportional to the amount of cell-elongating compound present.

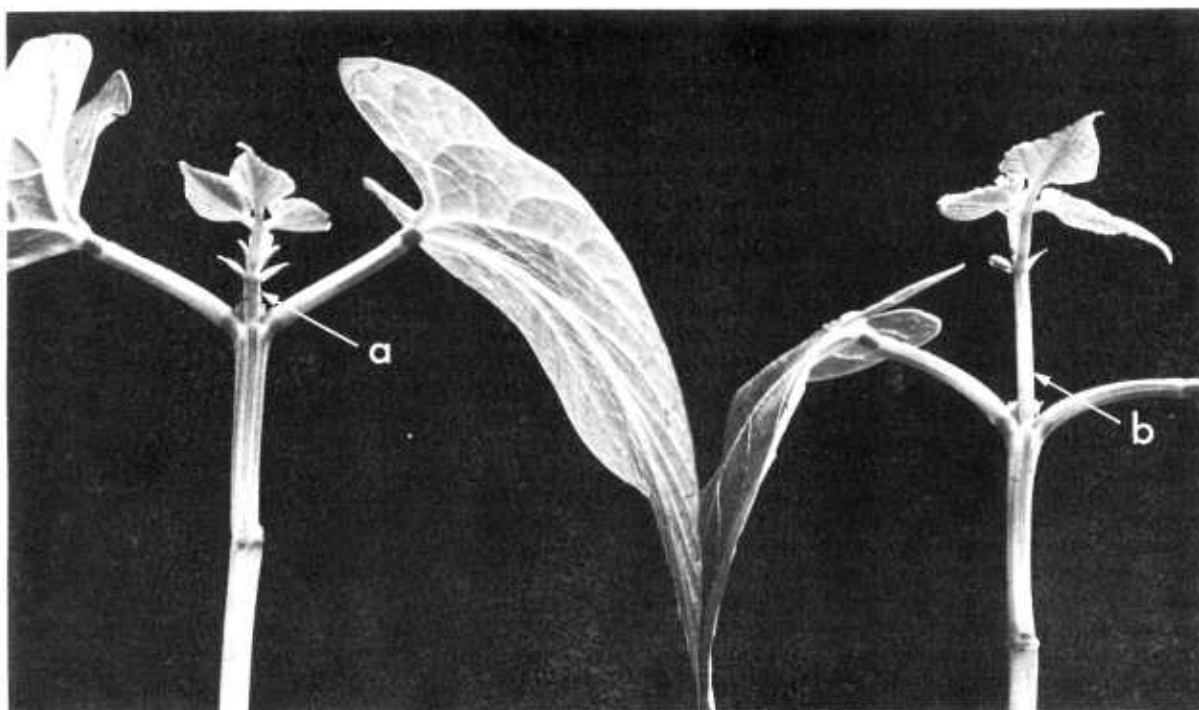
This method, which involves intact plants, is also useful for studying effects of extremely small amounts of compounds of the gibberellin type (fig. 9). It can also be used to detect compounds that retard elongation and those that influence cell multiplication in stem tissues.

Apparatus, Chemicals, and Other Materials

- Analytical balance
- Tweezers
- Dissecting microscope
- Shell vials
- Fractionated lanolin
- Water bath with temperature control
- Standard microscope slides
- Melting-point tubes
- Aquarium pump or compressed air
- Razor blades
- Glass rod pointed to about 0.5 mm. diameter
- Room with 700-foot-candle illumination from Slimline fluorescent tubes, photoperiod control, and temperature of 22°–24° C.
- Gibberellic acid for standard treatment

Suggested plant material—Young bean plants (*Phaseolus vulgaris* L., Pinto var.) with primary leaves only partly unfolded and with terminal buds having the tip of the center leaflet in the first trifoliate leaf slightly separated from the 2 lateral leaflets. The second internode should not be more than 1 mm. long at this stage of development. Discard plants with second internodes longer than 1 mm.

Preparation and selection of plant material—Select plants of uniform size and in the required stage of development. With pointed tweezers, remove the bract that subtends the dorsal side of the center leaflet in the terminal bud so that a smooth, well-defined scar is left and one side of the second internode is thus exposed. Measure the distance from the lower edge of the scar to the base of the bracts on the third node (fig.



BN-28950

FIGURE 9.—Comparison of length of bean second internode (a) treated with 250 micrograms of fractionated lanolin with length of second internode (b) treated with an equal amount of fractionated lanolin containing 63 trillionths of a gram of gibberellic acid A₃. Photo taken 4 days after treatment.

10). Record this as the initial length of the internode before treatment.

Procedure—After eluting a segment of a paper chromatogram or extracting the powder from a thin-layer plate containing the chromatographed compound, place the eluate in a small shell vial, evaporate the solvent, and add 1 ml. of ether containing 250 micrograms of fractionated lanolin. (See “Fractionated lanolin as a carrier for growth-regulating compounds,” p. 104.) Dissolve the solids in the vial by stirring and slightly warming the mixture, if necessary. Transfer the ether mixture from the vial to a small area on the surface of a microscope slide. To make this transfer, dip a capillary melting-point tube into the ether mixture and allow the liquid to rise in the tube by capillarity. Use the tube as a delivery pipette and place the ether mixture dropwise on the slide in an area directly under a stream of gently flowing air. In this way evaporate the ether leaving the compound to be tested thoroughly mixed with the fractionated lanolin.

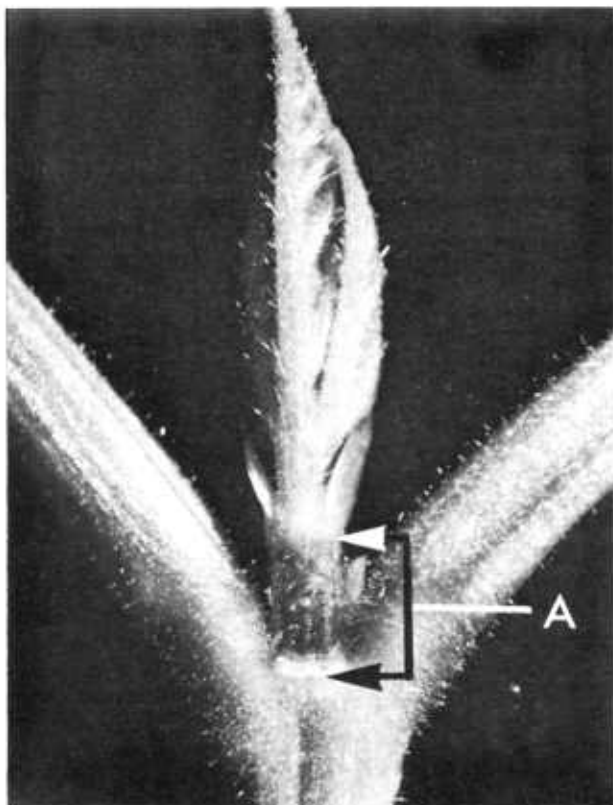
Remove the lanolin mixture quantitatively from the slide with a razor blade in a way that leaves the paste piled on the beveled edge at one end of the blade. With the aid of a dissecting

microscope, use the narrow glass rod to remove the lanolin mixture from the blade and place it on the second internode of one test plant (fig. 11).

If the cell-elongating properties of the chemical are to be studied, grow the plants in 700 foot-candles of light from Slimline fluorescent tubes for 9 hr. daily followed by darkness. Maintain the plants at 20°–23° C. during both the light and dark periods. If growth inhibition is to be studied, add about 50 foot-candles of illumination from Mazda lamps and increase the temperature to make the test plants elongate the desired amount. If maximum stem elongation is required, use about 50 foot-candles of light intensity from Mazda lamps for 2 hr. in the middle of the 15-hr. dark period.

Method of taking results—At the end of 4 days, measure the length of the second internode of each plant as described under “Preparation and selection of plant material.” Subtract the initial length of each internode and compare the growth with that of plants treated with the fractionated lanolin alone.

Since the test plants are exposed to greenhouse conditions during their early growth, their sensitivity to cell-elongating substances



BN-28933

FIGURE 10.—Terminal bud of young bean plant showing scar left after bract has been removed and the distance measured to determine length of second internode (A).

varies from experiment to experiment, depending on the prevailing light intensity. For example, plants grown during partly cloudy weather are generally more responsive than are those grown in bright sunlight during clear weather. However, even the relatively insensitive ones are capable of responding to extremely small amounts of gibberellic acid and other cell-elongating substances.

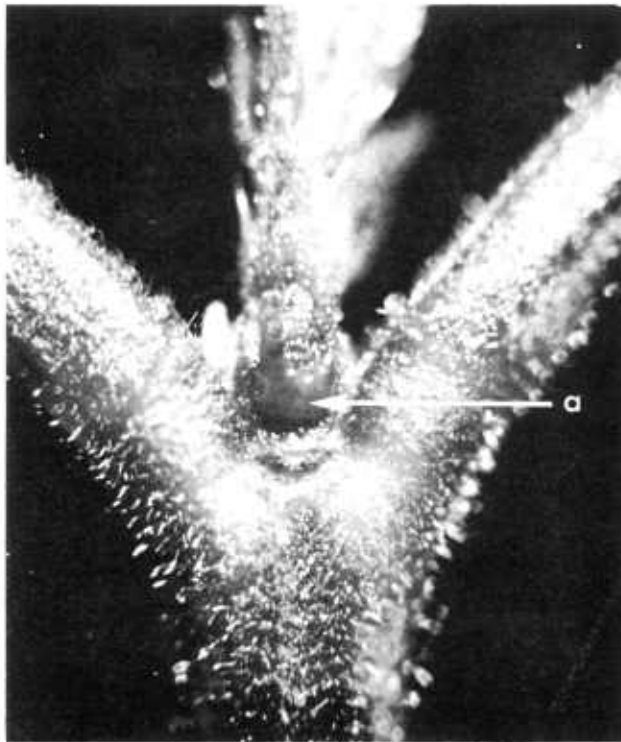
Suggested standard for comparison—Gibberellic acid applied at a dosage of 0.001 microgram or less per second internode.

A convenient way to obtain 0.001 microgram of gibberellic acid (1 billionth of a gram) is as follows: dissolve 2 mg. of gibberellic acid A_3 in 200 ml. of ethyl alcohol and designate this Solution I. Mix 1 ml. of Solution I with 99 ml. of ethyl alcohol to make Solution II. Finally, mix 0.5 ml. of Solution II with 2 ml. of ethyl alcohol to make Solution III. Place 0.05 ml. of Solution III (which contains 0.001 micrograms of gib-

berellic acid) in a shell vial. Evaporate the alcohol in a water bath at 50° C. Add 1 ml. of ethyl ether containing 250 micrograms of lanolin fraction. (See "Fractionated lanolin as a carrier for growth-regulating compounds," p. 104.) Stir until the gibberellic acid is thoroughly dissolved. Transfer the lanolin fraction-gibberellic acid mixture to the surface of a clean slide using a melting-point tube pipette and a gentle stream of air to evaporate the ether as described above. Apply the residual lanolin mixture of gibberellic acid to second internodes of bean plants as described in the above assay. If less than 0.001 microgram of gibberellic acid is desirable as a standard, extend the dilution series described and carry out the second internode test. When gibberellic acid is to be used repeatedly as a standard, prepare a large number of slides containing the lanolin fraction-gibberellic acid mixture as described above, and store these in a slide box at 0° C. for use when needed.

Reference

Method submitted by J. W. Mitchell and Greta D. York, Agricultural Research Service, U.S. Dept. Agr.



BN-28947

FIGURE 11.—Mixture of 250 micrograms of fractionated lanolin and a plant extract (a) applied to second internode of a test plant after bract at base of internode had been removed.

OAT FIRST INTERNODE METHOD

Based on research by J. P. Nitsch and Colette Nitsch; D. G. Crosby, R. V. Berthold, and Roy Spencer, Jr.

Description of method—Segments 4 mm. long of first internodes of etiolated oats are incubated 20–24 hr. with the solution to be assayed. The increase in length is proportional to the logarithm of the concentration, at least between limits. The test is sensitive to 0.001 microgram of indole-3-acetic acid or gibberellic acid A₃. This method is also useful for assaying paper chromatograms.

Apparatus, Chemicals, and Other Materials

Plastic containers, about 30 × 20 × 10 cm., with lids
Vermiculite or clean sawdust
Incubator at 25° C. ± 0.5° C.
Dark chamber with green light (about 540 millimicrons)
Cutter with 2 parallel razor blades and a guide set to cut segments 4 mm. ± 0.05 mm. long (See "Segment cutters," p. 101.)
Analytical balance
Petri dish with gauze or muslin stretched over the top
Paper towels
Forceps
Fine scissors
Test tubes, about 13 mm. diameter
Roller-tube apparatus (rotating at 1 r.p.m.)
Binocular microscope (10×) with ocular micrometer
Stainless steel spatula
A fraction of a milligram of each compound to be evaluated
250 mg. gibberellic acid A₃ or indole-3-acetic acid for standard treatment

Suggested plant material—Seeds of oat (*Avena sativa* L., Brighton var.). These can be obtained for research from: (1) Cereals Department, Canada Department of Agriculture, Experimental Farm, Scott, Saskatchewan, Canada; (2) O. Gelin, Plant Breeding Institution, Weibullsholm, Landskrona, Sweden; (3) Small Grains Collection, Cereal Crops Research Branch, U.S. Department of Agriculture, Plant Industry Station, Beltsville, Md. 20705.

Preparation and selection of plant material—Place a 2.5-cm. layer of moist vermiculite or sawdust in plastic boxes. Sprinkle over

it the Brighton oat seeds. Cover with another 2.5-cm. layer of the planting medium. Close the lid tightly as an increase in CO₂ enhances the growth of the first internodes. Place the boxes in the incubator at 25° C. and keep them in *total* darkness. (Neither seedlings nor segments should ever be exposed to anything more than the minimum amount of dim green light required for perception.) The seedlings are ready for use 64 hr. later.

Procedure—Prepare the following buffer solution:

K₂HPO₄:1.794 g./liter
citric acid monohydrate:1.019 g./liter
sucrose:20 g./liter

Into each test tube, place 0.5 ml. of this solution in which may be incorporated the extracts or chemicals to be tested.

The method is especially suitable for assaying paper chromatograms. Use paper approximately 2 cm. wide to develop the chromatogram. Cut the chromatogram horizontally into 1-cm. strips. Make certain that each strip is numbered starting with the segment nearest the origin of the chromatogram. Place each piece of the chromatogram separately into a test tube containing 0.5 ml. of the buffer solution, leaving 2 tubes with paper segments cut below the starting line and above the front of the chromatogram to serve as a control.

In a dark room with the dim green light, harvest the seedlings, breaking them at the seed. With fine scissors, remove the coleoptile by cutting exactly at the node. Place the first internodes on wet paper towels, orienting the apical (nodal) ends uniformly.

With the cutter using parallel razor blades, cut from each first internode one 4-mm. segment 2 mm. below the node. (See "Segment cutters," p. 101.) Wash the segments by placing them for 1 hr. on a piece of muslin stretched over a petri dish filled with glass-distilled water. The segments are partially immersed in this way but are exposed continuously to air. The muslin prevents the segments from sinking into the water.

Transfer 10 segments to each of the test tubes using a thin spatula. Place tubes in the roller-tube apparatus and rotate them at 1 r.p.m. for 20–24 hr. in darkness at 25° C. and in an atmosphere where the humidity is near saturation. If a high humidity cannot be obtained, seal the tubes with Parafilm.

Method of taking results—After the incubation period, remove the segments from the tubes with a wire loop and place them on microscope slides. Measure the length of the segments to the nearest 0.1 mm. under a binocular microscope

with an ocular micrometer. Compare the average length of each of the 10 segments in each tube with curves based on response to different amounts of the standard compounds, plotted on semi-logarithmic paper.

Suggested standard for comparison—Either indole-3-acetic acid or gibberellic acid A₃ at concentrations of 1, 3, 10, 30, and 100 micrograms/liter.

Other compounds active when used in this method are: auxins, such as the amide, nitrile, and esters of indole-3-acetic acid; *cis*-cinnamic acid (above 300 micrograms/liter); various growth-regulating substances; gibberellins A₁, A₂, A₄, A₅, A₆, A₇, A₈ (above 10⁻⁸ M), A₉, but not steviol; helminthosporol; helminthosporic acid; and dehydrohelminthosporic acid. The last 3 compounds have a relatively low activity.

Variation used by Crosby, Berthold, and Spencer—First internode segments from 7 varieties of oats were compared with respect to sensitivity and uniformity of elongation response to different growth-regulating substances. The method used in these comparisons was essentially the same as that described above. The Forkeddeer variety was most suitable for obtaining first internode segments with which to measure the effect of regulating substances and extracted plant hormones on elongation of segments from oat first internodes. Small amounts of Forkeddeer seed oats to be used for scientific investigations are obtainable from: (1) Farmers Supply Co., Smithville, Tennessee 37166; (2) D. R. Mayo Seed Co., Box 10247, Knoxville, Tennessee 37919; and (3) J. M. McCalla, Jr., Covington, Tennessee 38019.

References

- Crosby, D. G., Berthold, R. V., and Spencer, Roy, Jr. Naturally Occurring Growth Substances. II. An Improved Straight Growth Test and Its Applications. *Plant Physiol.* 36: 48–51. 1961.
- Nitsch, J. P., and Nitsch, Colette. Studies on the Growth of Coleoptile and First Internode Sections. A New, Sensitive, Straight-Growth Test for Auxins. *Plant Physiol.* 31: 94–111. 1956.
- Nitsch, J. P., and Nitsch, Colette. Comparison of Activities of Nine Gibberellins by Three Biological Tests. *Ann. Physiol. Vég.* 4: 85–97. 1962.

PEA STEM SECTION METHOD

Based on research by G. S. Christiansen and K. V. Thimann.

Description of method—The effect of a compound on stem elongation is measured by floating segments from etiolated stems of pea on the

surface of a solution containing the compound, and measuring the response in terms of increase in length or weight of the segments.

Apparatus, Chemicals, and Other Materials

Dark room with temperature controlled at about 27°–29° C. and humidity controlled at 85–90%

Analytical balance

Glass or metal containers

Corning Signal Red filter and 60-w. lamp

Quartz sand

Razor blades mounted 20 mm. apart on a block of wood (See "Segment cutters," p. 101.)

Beakers, 500 ml.

Filter paper

Petri dishes

Sodium hydroxide

1 g. of indole-3-acetic acid

250–500 mg. of each compound to be tested

Suggested plant material—Etiolated stems of pea (*Pisum sativum* L.)

Preparation and selection of plant material—Soak the seeds in tapwater for 6–8 hr.; plant them in sand moistened with tapwater, and allow the plants to grow in darkness. All observations and manipulations should be made in a minimum of light from a 60-w. incandescent lamp passed through the Corning filter. After 7 days, select 30–50 plants of uniform size from which to make stem segments.

Procedure—Do not use plants that have developed fourth internodes longer than 3 mm. Cut 20-mm. segments (exactly measured) from the third internodes. In cutting segments, first decide which portion of the stem is most suitable for the test at hand. Purves and Hillman showed that segments from an area farthest from the apex respond least to gibberellic acid and sucrose, but these segments show a relatively great difference in response to indole-3-acetic acid as compared with gibberellic acid. Short segments taken from the stem close to the apex are relatively responsive to gibberellins. Therefore, select an appropriate portion of the stem from which to obtain segments and, in reporting results, indicate its location.

Immediately place the segments in tapwater, wash, and divide them into groups of 15 segments each. Blot dry with filter paper and weigh the groups separately to the nearest milligram. Keep segments moistened.

Prepare aqueous solutions (25–100 ml.) of the compounds to be tested, adjusting their pH

to 6.0 with the NaOH, and add the KCl to each to avoid variation in potassium content of the solutions. Store the solutions in a refrigerator if they are to be used repeatedly and make up new ones every few weeks if they are to be used for an extended period. Concentrations ranging from 0.1–2.0 mg. per liter of distilled water are suggested.

Place the solution containing the compound to be tested in a petri dish and float a weighed group of 15 segments on the surface of the liquid for 24 hr. in darkness.

Method of taking results—At the end of 24 hr. remove the segments, blot them with filter paper, weigh them as a group to the nearest milligram, and measure their length individually to the nearest millimeter. Compare the weight increase and average length increase of these segments (from each concentration of the test chemical) with comparable measurements of segments treated with distilled water only, and of others treated with distilled water containing different concentrations of indole-3-acetic acid.

Variations of the above method and the effect of various experimental conditions on the response of pea stem segments to growth substances were studied in detail by Galston and Hand.

Suggested standard for comparison—Solution containing 0.1, 0.5, 1.0, and 2.0 mg. of indole-3-acetic acid per liter.

References

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RADISH COTYLEDON METHOD

Based on research by Tetsuo Takematsu.

Description of method—The increased angle that develops when cotyledon and cotyledonary petiole are sprayed with an aqueous solution of a compound indicates the influence of the compound on cell elongation.

Apparatus, Chemicals, and Other Materials

Composted soil
Clay pots, 9 cm. in diameter
Scissors or razor blade
Small glass tubes or pipettes
Analytical balance
Atomizer
Protractor
Photographic paper and developer
Glass plate
Waterproof cellophane
Distilled water
50 mg. of compound to be tested
5 mg. of alpha-naphthaleneacetic acid for standard treatment

Suggested plant material—Seedlings of Japanese radish (*Raphanus sativus* L.)—Tokinashi Daikon variety, or any other variety that develops relatively large and persistent cotyledons. The variety suggested is especially sensitive to growth-regulating substances; the size and shape of the cotyledon and cotyledonary petiole form angles that are easy to measure; and the cotyledon is dark green and vigorous, providing uniform plant material throughout the year. Other varieties with comparable characteristics can also be used, but it may be necessary to determine the responsiveness of these under the conditions available.

Preparation and selection of plant material—Select mature, round, fresh seeds. Make 10 holes 0.5 cm. in diameter and 1 cm. deep in the soil in each pot. Plant 2 seeds in each hole. Place the pots in sunlight or bright light and maintain the humidity at about 60–80%. Select 20 seedlings of uniform size 4–8 days after germination. Use 10 plants for the application of each compound to be tested and 10 as controls.

Procedure—Dissolve each compound in distilled water using the following concentrations: 1, 10, 50, 100, and 200 p.p.m. Spray 1–5 ml. of the solution to be tested on the whole upper surface of the 10 seedlings. Spray 10 control seedlings with a corresponding amount of distilled water. Place the plants in a dark room for 24 hr. After the cotyledons and cotyledonary petioles have developed curvature, sever the aboveground portion of the plants at the base of the stem with scissors or a razor blade. Cut off the cotyledons at the point where they join the cotyledonary petioles. Make shadowgraphs of petioles and stem segments to record the curvature of the petioles. Blueprint paper or other light-sensitive paper can be used to make shadowgraphs. Place a sheet of waterproof cellophane over the sensitive paper and arrange

the pieces of plant to be shadowgraphed on the cellophane. Lay a glass plate on the plant samples so that undistorted shadowgraphs can be made by means of the proper light exposure and then measure the angle y (fig. 12).

For an alternate method place the petiole and stem segment on heavy paper; with pins mark the crossing point (fig. 12, *b*) of the cotyledonary petioles and the 2 points at which the cotyledonary petioles begin to bend downward (fig. 12, *a* and *c*). A line drawn to connect these 3 pinholes indicates angle y .

Method of taking results—Measure the angle y on treated and untreated petiole and stem segments. The difference of the angle between treated and untreated segments indicates the cell-elongating property of the compound applied. In general, this angle on untreated segments is about 20° , depending on the humidity of the soil and the amount of light. It is important to subject both the plants to be treated and those to be used as controls to the same growing conditions.

Suggested standard for comparison—0.1–50 p.p.m. solution of alpha-naphthaleneacetic acid or 2,4-dichlorophenoxyacetic acid.

Reference

Takematsu, Tetsuo. New Methods for the Qualitative and Quantitative Assays of Growth Substances; Research on the Raphanus Test and Its Application. Utsonomiya University, College of Agriculture. Gakujutsu Hokoku Tokushu. Spec. Bul. 12, 193 pp. 1961. [In Japanese.]

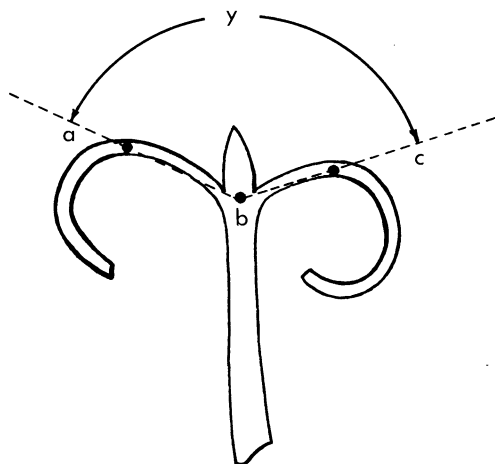


FIGURE 12.—Method of measuring angle y , formed by intersecting lines drawn through points *a*, *b*, and *c* on the stem and petioles of radish seedling to show response to growth substance. (Diagram by Tetsuo Takematsu.)

SPLIT PEA STEM METHOD

Based on research by Martha Kent and W. A. Gortner; K. V. Thimann and C. L. Schneider; F. W. Went and K. V. Thimann.

Description of method—The angle of curvature that develops when split stems of pea are immersed in an aqueous solution of a compound indicates the influence of the compound on cell elongation.

This test has been carefully standardized and widely used in measuring cell-elongation properties of synthetically prepared regulators and regulating compounds obtained from plant material.

Apparatus, Chemicals, and Other Materials

Quartz sand
Completely darkened room with temperature controlled at 27° – 29° C.
Ruby glass incandescent lamp
Time clock
Razor blades or segment cutter (See "Segment cutters," p. 101.)
Analytical balance
Petri dishes, 10 cm.
Several beakers, 500-ml.
Protractor
Photographic paper and developer, if shadowgraphs are to be made to record stem curvatures
20–50 mg. of each compound to be evaluated
20 mg. of indole-3-acetic acid for standard treatment
Sodium hydroxide
Potassium chloride
Distilled water

Suggested plant material—Stem segments of etiolated pea plants (*Pisum sativum* L.)—Alaska variety.

Preparation and selection of plant material—Soak pea seeds in water for 6 hr.; then plant them in moist sand in a container that allows for drainage, and keep them in complete darkness. Increase sensitivity by daily exposure to 4 hr. of red light from a 25-w. ruby bulb about 61 cm. away. If an automatic timer for the light is not available, use a single exposure to red light 32 hr. before harvest.

Harvest the plants under ordinary room light after 7 or 8 days, when the fourth internode is less than 5 mm. long. Use plants with a third internode 4–7 cm. long and cut a 3.5-cm. segment 5 mm. below the top of the third internode. With a razor blade, split the segments

centrally for three-fourths of the length downward and drop the segments into water. The segments may be washed for several hours in tapwater but some investigators have found this unnecessary. Discard the seeds and roots.

Procedure—Prepare several dilutions (distilled water) of the compound to be tested (10^{-7} – 10^{-3} M) and place 10 ml. of each dilution in a separate petri dish. These solutions should not be more acid than pH 4. Transfer 6 or 8 of the split segments to each dish and leave them for 6–24 hr. in darkness. Curvature will be complete at the end of 6 hr. and will not change.

In an alternative test, split oat coleoptiles are used in place of split pea stems. Grow the plants (Victory variety), as described for peas, for 76 hr. Decapitate the coleoptiles at a distance of 2–4 mm. from their tips, and split each one lengthwise with a razor blade for a distance of 20 mm. from the cut surface. Cut the coleoptile off a few millimeters below the split and wash the split segments in tapwater. Discard the seeds and roots.

Place not more than 8 of the segments in 20 ml. of the solution to be tested. Use petri dishes to contain the test solution and adjust its pH to 5.5 with sodium hydroxide. Add 0.001 M potassium chloride solution, which is known to increase slightly the effect of auxin.

Some researchers find improved response using a pH 7.0 phosphate buffer included at 3×10^{-3} M in each dilution tested. If compounds are difficult to dissolve, 0.15 ml. of 2.5% sodium hydroxide or 0.5 ml. of 95% ethanol may be added to a 20-mg. sample before making to volume.

To obtain the response, place 5 pea segments in 25 ml. of test solution in each petri dish overnight in the dark at room temperature.

Method of taking results—Lay the stem segments on photographic paper (protect the paper from moisture with a piece of glass), expose the segments and paper to light, and develop the image on the paper using usual photographic methods. Draw lines parallel with the tip and base of one curved, split portion of segment. Measure the angle a with a protractor. Draw similar lines using the opposite segment, and measure the angle that corresponds to a . Most regulating compounds having cell-elongating properties induce a decrease in the angle indicated compared with that formed by the segment when placed in distilled water. The angle measurement illustrated is one of several that have been utilized. For a general discussion of angle measurements and their relation to the amount and kind of regulating chemicals used, see references listed at the end of this method.

Measure the angle a on each segment with the protractor (fig. 13) and average the measurements. If curves are needed to compare observations, plot the average values against the logarithms of the concentration. A straight line will be obtained within certain limits of concentration. Use the slope of the line to compare the activity of different compounds by the formula:

$$a = \frac{C}{K \log C_0}$$

Where a is the mean observed curvature

C the concentration (moles per liter)

C_0 the concentration at which the curvature is 0

K the slope of the line

Use at least 3 points to characterize the slope of the line.

If split coleoptile segments are used, measure the angles that develop during the 24-hr. period immediately following the immersion of the segments in the test solution and compare the activity of different compounds.

The percent relative activity is obtained by comparison of curvatures to a reference standard such as indole-3-acetic acid or alpha-naphthaleneacetic acid at the corresponding concentration. The percent relative activity is calculated by dividing the average curvature of the test compound by that of the reference compound and multiplying by 100. The average curvature for each compound is found by measuring both arms of 5 split segments and dividing by 10. The angle of curvature is measured at the point of inflection between the inward and outward curvature (angle a in fig. 13).

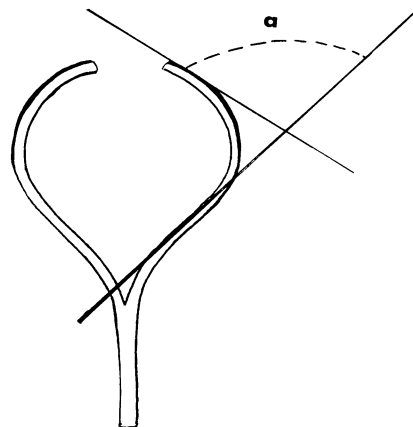


FIGURE 13.—Shadowgraph of split segment of pea stem showing curvature that developed upon exposure to growth-regulating chemical and method of measuring angle of curvature (a). (Diagram suggested by F. W. Went.)

The concentrations most suitable for the testing of synthetic growth regulators are 10 and 100 p.p.m. or 10 and 200 p.p.m. Compounds vary as to the concentration for optimum curvature and toxicity is evidenced by limpness of the split segments.

Suggested standard for comparison— 10^{-3} – 10^{-6} M indole-3-acetic acid.

References

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TOMATO HYPOCOTYL METHOD

Based on research by G. F. Pegg; I. D. J. Phillips and R. L. Jones.

Description of method—Segments of etiolated tomato hypocotyls are incubated with solutions of compounds to be tested while revolving slowly on a clinostat. Growth inhibition is assessed by comparing the linear extension of the test segments with that of the controls.

This test can also be modified to measure activity of gibberellins by incubating the hypocotyl tissue in white light during the assay.

Apparatus, Chemicals, and Other Materials

- Glass petri dishes, 10 cm. in diameter
- Seed germination disks, 10 cm.
- Humidity box with standing grill over free water surface, approximate relative humidity 90%
- Light-proof incubator with uniform temperature of 25° C.
- Dark room
- Red light source, Osram 6 R.K. 60-w. tungsten lamp
- Forceps
- Sheet of glass marked with 1-cm. divisions
- Magnifying lens or bench binocular microscope
- Single-edge razor blades
- Camel's-hair brush
- Glass combustion tubes with internal diameter less than 10 mm., 75 × 8 mm.
- Plastic or rubber caps
- Strips of Whatman No. 1 chromatographic paper, 64 × 13 mm.

Automatic pipette delivering 1.5 ml. solution

Analytical balance

Clinostat with drums to hold tubes

Constant temperature room with continuous light at 700 foot-candles (warm white 80-w. fluorescent tubes)

Glass graticule with 1-mm. divisions

pH meter

Deionized water

250 mg. of 2,4-dinitrophenol, coumarin, or gibberellic acid A₃ for standard treatment

M/200 potassium hydrogen phosphate, citric acid buffer at pH 7.0

1.0 g. of 2,4-dinitrophenol

0.5 g. of coumarin

0.5 g. of gibberellic acid A₃

Suggested plant material—Seeds of tomato (*Lycopersicon esculentum* Mill. cultivars Potentate and Ailsa Craig.)

Preparation and selection of plant material—Soak virus-free tomato seeds for 3 hr. at 25° C. in deionized water. Sow 80–90 seeds on a seed-germination disk in a petri dish. Sow seeds in as many of these dishes as are required. Irrigate each germination disk with 1.5 ml. deionized water. Place the dishes in a humidity box maintained at about 90% relative humidity and incubated at 25° C. for 116 hr. in total darkness. The etiolated seedlings will then be 12–15 mm. high. While using red light, remove seedlings of uniform size from the dishes with forceps and place them on a sheet of wet glass. Use a magnifying lens or a bench binocular microscope and a razor blade to remove carefully the apical meristem and cotyledons. Take a 1-cm. segment, measured from the apex of the hypocotyledonary arch to the taproot. This leaves a crozier-shaped segment (fig. 14) with the terminal portion of the hook about 3 mm. long. Float the segments on distilled water in the dark for not more than 2 hr. until the test is ready to be set up.

Procedure—Using red light, remove washed segments from the distilled water with a paint brush. Place 2 segments in a glass combustion tube containing a strip of Whatman No. 1 chromatographic paper washed with acetone and then thoroughly dried. The filter paper strip is included to reduce variables when chromatographed plant extracts and known regulating substances are compared. Pipette automatically into the tube 1.5 ml. of the solution to be tested, made up in 200 M potassium hydrogen phosphate-citric acid buffer at pH 7.0. (See buffer table, p. 129.) Place a plastic or rubber

cap over the end of the tube. Place the tubes in racks and revolve them at 2 r.p.m. on a clinostat in a dark incubator for 48 hr. In a similar way, set up segments for controls in plain buffer solution.

When working with crude plant extracts, chromatograph once or twice, the second time with solvent ascending on Whatman No. 1 papers 25.4 cm. square. Cut the paper into 20 strips of equal width (12.7 mm.), cutting parallel to the line on which the samples were spotted. Number each strip, starting at the end where the samples were spotted. Divide each strip into 4 segments each 63.5 mm. long and number these 1A, 1B, 1C, 1D, etc.; 2A, 2B, 2C, 2D, etc., making a total of 80 segments of the chromatogram. Fit each of these into a combustion tube (described above). Add 1.5 ml. of the pH 7.0 buffer solution and 2 of the plant segments to each tube. In this way the chromatographed substances are eluted into the buffer solution during rotation on the clinostat simultaneously affecting the growth response of the hypocotyl segments.

It is imperative that all preparatory work and subsequent incubation be carried out in air

that is not contaminated with fuel gas, i.e., in a room free from gas fittings.

Where the test is designed to measure growth-promoting activity of solutions of gibberellins or of chromatographed plant extracts containing gibberellin-like substances, set up the plant segments as described, but incubate them on the clinostat under continuous white light of approximately 700 foot-candles using warm white fluorescent tubes. A disadvantage of this particular assay is that naturally occurring inhibitors must be removed first or they will mask any gibberellin growth response.

Method of taking results—Carefully remove the elongated segments from the tubes with forceps. Place them on blotting paper and measure to the nearest 0.5 mm. against a glass graticule. Calculate inhibition or stimulation as follows:

$$\frac{\text{Final length of test segment}}{\text{Final length of control segment}} \times 100 = \% \text{ growth inhibition or stimulation}$$

The results may be conveniently expressed as a histogram.

Suggested standard for comparison—Standard treatment for growth inhibition, for example, between 1×10^{-7} and 1×10^{-3} M. 2,4-Dinitrophenol or 1×10^{-7} – 1×10^{-2} M coumarin. For growth promotion, use 1×10^{-5} M gibberellic acid A₃. Noninhibited control segments usually undergo a threefold extension in buffer solution.

Variation used by Phillips and Jones—Soak dwarf pea seeds, Meteor variety, for 8–12 hr. in tapwater and then sow them 6–8 cm. deep in damp vermiculite contained in boxes. After germinating the plants for 4 days at 25° C. in darkness, sever the epicotyls from the cotyledons and roots and wash the epicotyls with distilled water. Pour 4 ml. of the test solution into each of a number of small glass vials. Stand 10 epicotyls upright in each of the vials. Use a comparable setup with distilled water instead of test solution for controls. Illuminate the epicotyl segments with light from Daylite fluorescent tubes at 20° C. for 3 days. Determine the mean length of the epicotyls above the first node for segments in each individual tube. Regard the initial length of each epicotyl above the first node as zero and use the final length above the node as a measure of absolute growth over the 3-day incubation period.

This test is sensitive to a concentration of 10^{-4} mg. of gibberellic acid/liter of water. Under these conditions, the segments are insensitive to indole-3-acetic acid and there is no additive or synergistic interaction between indole-3-acetic acid and gibberellic acid.

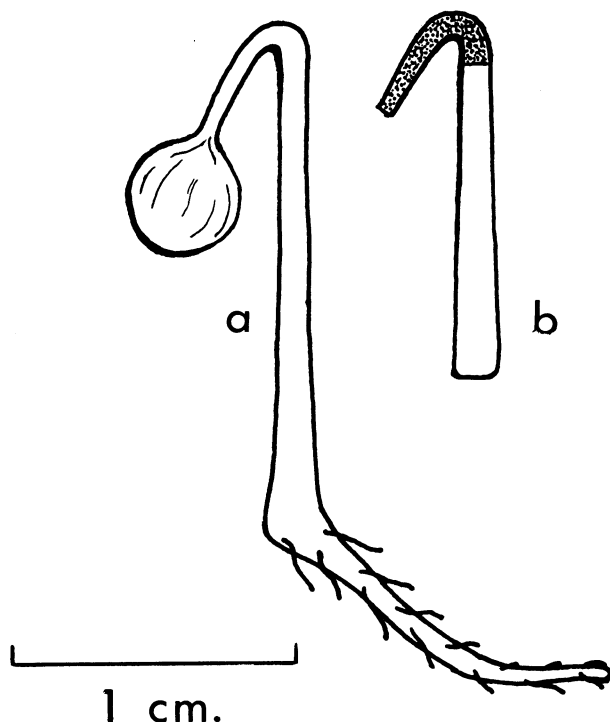


FIGURE 14.—Etiolated tomato seedling (a) 48 hr. after germination, showing only part of the taproot; hypocotyl segment (b) prepared for bioassay with root and cotyledons removed. Shaded portion denotes meristematic region. (After diagram by G. F. Pegg.)

If paper chromatography is used in conjunction with this test, elute the desired segments of the paper with water and subject the eluate to the pea epicotyl test without placing the paper segment in the tube. If thin-layer chromatography is used, place the silica gel containing a separated component in the tube with water and the epicotyls.

The difference in the length of the treated and control epicotyls at the end of 3 days is used as a measure of activity of the sample in terms of cell elongation.

References

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WHEAT COLEOPTILE STRAIGHT GROWTH METHOD

Based on research by F. Wightman; variations by C. R. Hancock, H. W. B. Barlow, and H. J. Lacey; G. F. Pegg; and J. van Overbeek and L. Dowding.

Description of method—The effects of compounds on cell elongation are measured by floating segments of wheat coleoptiles on aqueous solutions of the chemical and comparing the length of these with the length of segments floated on distilled water.

Apparatus, Chemicals, and Other Materials

Filter paper, Whatman No. 3, 10 cm. in diameter

Petri dishes, 10 cm. in diameter

Table space in a room with the temperature controlled at 24°–26° C.

Constant humidity boxes with glass covers

Kodak Ruby Signal light filter or red incandescent lamp (50 w.)

Razor blades and cutting instrument. (See "Segment cutters," p. 101.)

Small forceps or paint brush

Needle

Analytical balance

50–100 mg. of each compound to be evaluated

50–100 mg. of sodium 2,4-dichlorophenoxyacetate for standard treatment

Glass-distilled water

Suggested plant material—Germinating seeds of wheat (*Triticum aestivum* L., also called *T. sativum* Lam., and *T. vulgare* Vill.) Eclipse variety. Other varieties of wheat may also be used for this test.

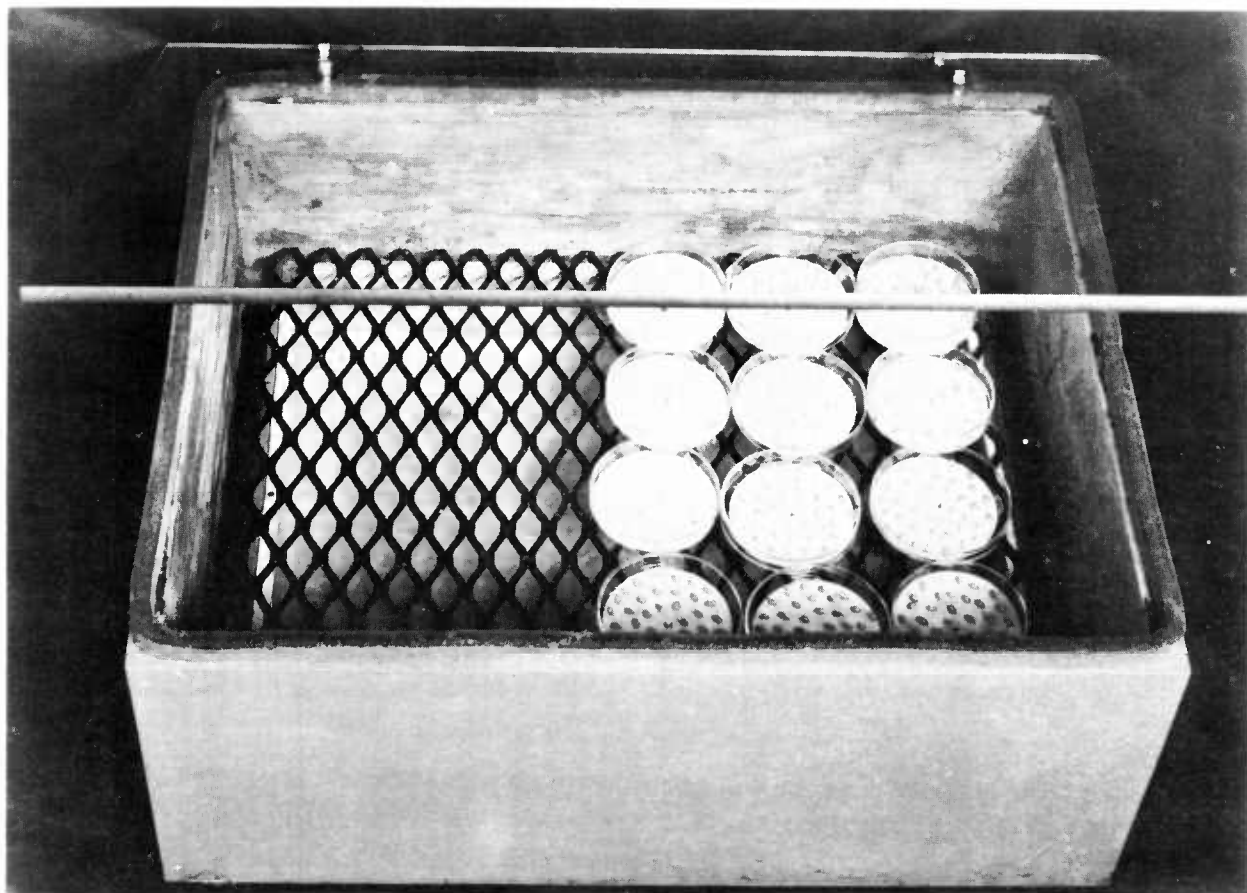
Preparation and selection of plant material—Soak wheat seeds in distilled water for 2 hr. and then place them with embryos facing upward on moist filter paper in open petri dishes (30–40 seeds per dish). Allow the seeds to germinate for about 66 hr. at 24°–26° C. Maintain a humid atmosphere (80–90%) around the seeds by placing the petri dishes in a constant humidity box (fig. 15). Place the open dishes on a perforated metal shelf resting on the upper edges of a metal water-containing tray in the bottom of the box. During the first 50 hr. of growth, expose the seeds to light from the red incandescent lamp or to light passed through a ruby red filter. The red light should be about 60 cm. from the seeds. Allow subsequent growth of the plants to take place in darkness.

Approximately 66 hr. after placing the seeds on the moistened filter paper, select plants with coleoptiles 17–20 mm. long. Then, with the aid of the cutting instrument excise 1 segment 10 mm. long from the upper-middle region of each coleoptile. Use the segment cutter designed so the apical 3-mm. segment of each coleoptile is removed and left in the instrument, while the next 10-mm. segment of the coleoptile is excised by the razor blades and forms the test segment. Remove the primary leaf by threading the segments onto fine glass capillaries, 2 segments per capillary, and then allow the segments to float on distilled water in a petri dish until required for the experiment. This pretreatment period should not exceed 3 hr.

In other methods, the 15-mm. segment used as the test segment is measured, starting 2 mm. from the tip. The tip segment is again removed to eliminate, as far as possible, the effects of endogenous auxin.

Procedure—Prepare solutions representing a dilution series of the compound to be tested by diluting a concentrated stock solution of the compound with distilled water; suggested concentrations are 0.01, 0.1, 1, 10, and 100 p.p.m. Place about 20 ml. of each concentration in respective petri dishes and float 10 coleoptile segments on the surface of the liquid in each dish. Allow the segments to grow for 24 hr. in darkness at 24°–26° C. Float additional segments on distilled water and use these as controls.

Method of taking results—Remove segments from the petri dishes with small forceps or



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FIGURE 15.—Constant humidity box containing germinating wheat seeds in open petri dishes. Note water-containing tray at bottom of box and strips of soft rubber around upper edges of box to provide good seal with hinged plate-glass top. All inside surfaces of box are coated with several layers of paraffin wax. (Photo—courtesy of F. Wightman.)

paint brush and blot them dry. Measure each segment to the nearest 0.5 mm. under a glass graticule, or place these segments on a slide in a photographic enlarger and measure the projected shadow. Compare the mean elongation of segments in the different solutions with that of the control segments in distilled water. Express the results as a percentage. Thus,

$$\frac{\text{final length of treated segments (mm.} \times 100)}{\text{final length of water control (mm.)}}$$

Invert the slide of coleoptiles on the blades of the cutter (fig. 16), keeping the tips against the guide block, and then exerting a gentle downward pressure. (See also "Barlow cutter," p. 101.) Remove the unwanted 3-mm. tips thus cut from the coleoptiles by passing a needle along the outside of the 2 blades. Lift off the

10-mm. segments by inserting the needle between the blades and lifting with the slide. Retain only the cut segments that are completely filled with leaf tissue. Place the segments in bundles of 3 or 5 on moist filter paper in closed petri dishes, in chronological order, and store at 5° C.

Place 3 or 5 coleoptile segments and 0.5 ml. of solution to be tested in a specimen tube 50 × 8 mm., again using the coleoptiles in chronological order. Place these tubes in a rack (fig. 17). Place one or several of these racks filled with tubes in a clinostat (fig. 18). Rotate the tubes horizontally about their long axis for 17–20 hr. at 20° C. and at about 180 r.p.h. It is preferable to leave the tubes uncorked, in which case the open ends must be prevented from touching the ends of the frame or the base of the next rack. Remove racks of tubes from

clinostat in the constant-temperature room to the laboratory. Shake the coleoptile segments or draw them up with a needle onto the side of the tube, clear of the solution or chromatographic paper if present. Measure them directly through the wall of the tube with a ruler to the nearest 0.5 mm. and record the increase over the original length.

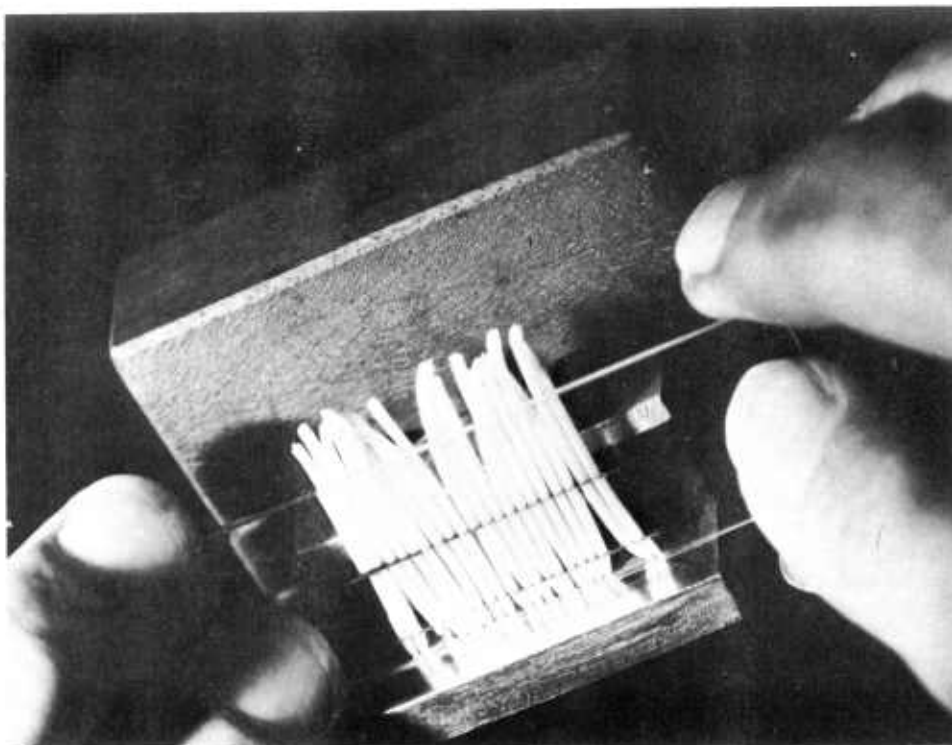
Van Overbeek and Dowding followed this same method of taking results but used segments of oat leaf bases for assaying gibberellins. The tubes are rotated for 48 hr. and the solutions preferably contain 2% sucrose.

Suggested standard for comparison—Solution containing 1 p.p.m. of sodium 2,4-dichlorophenoxyacetate.

Variation used by Hancock, Barlow, and Lacey—Obtain feldspar chips of a size that will pass through a 2.5-mm. mesh screen but will be retained by a 1-mm. mesh screen. Wash the feldspar and oven-dry it. Soak the seeds for 1 hr. in a shallow layer of tapwater at 20° C. Place a measured amount of the feldspar in an aluminum dish, add a measured quantity of seeds,

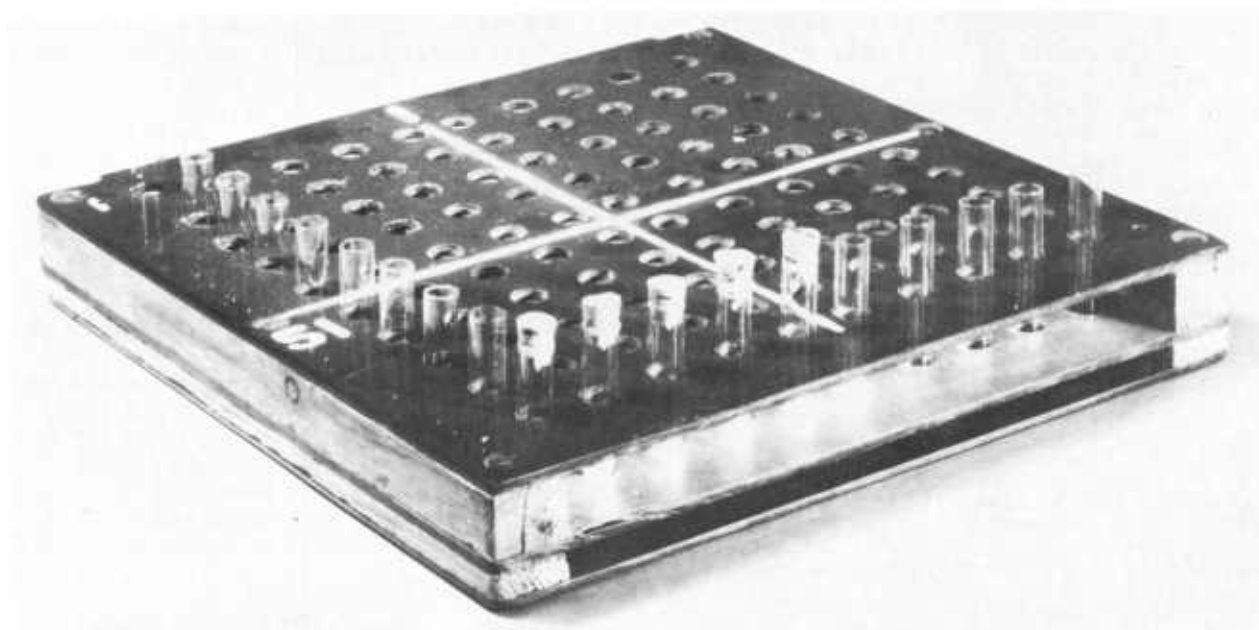
cover these with a measured amount of the feldspar to make a layer about 3 times the width of the seeds, and add a predetermined volume of water sufficient to moisten the feldspar. Cover dishes with glass sheets and maintain them at 20° C. for 80 hr. in darkness. Sow in white light but, for further manipulations, use phototropically inactive safe lights (60-w. bulb behind a Kodak filter OA or OB).

Remove the glass covers when the coleoptiles are just showing through the feldspar. Add sufficient water at room temperature to replace that evaporated, and then let dishes stand about 17 hr. until the coleoptiles are about 20–30 mm. long. Sever coleoptiles from the seeds with a scalpel and place them in rows on wet microscope slides, keeping them in the chronological order in which they were cut. Arrange coleoptiles at right angles to, and with their tips coincident with, one edge of the slide. Place the slide against the zero line of an aluminum grader scribed at 5-mm. intervals. Place coleoptiles with bases falling between any 2 adjacent lines in one length grade, maintaining chronological order, and “line them up” as before to cut a 10-mm. segment from them.



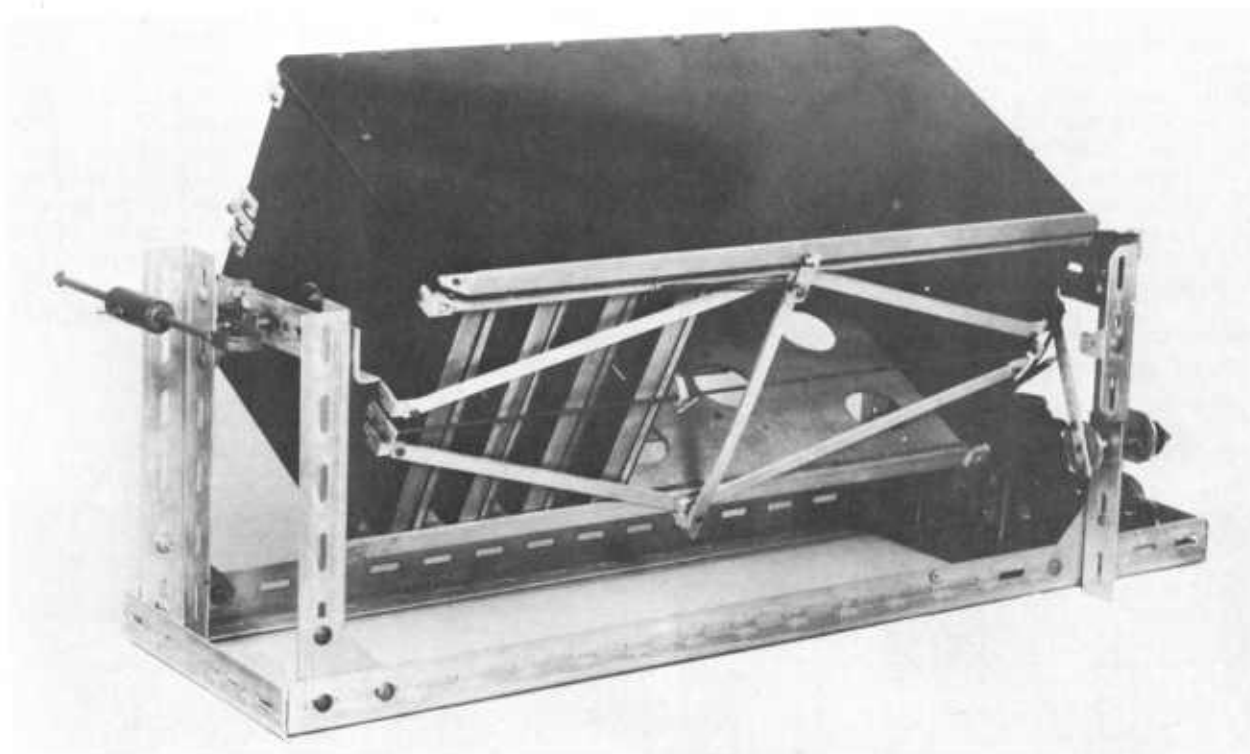
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FIGURE 16.—Segments of coleoptiles have been cut by pressing slide carrying 20 coleoptiles against 2 razor blades. Tips of the coleoptiles rest against the stainless steel guide block. (Photo—courtesy of Journal of Experimental Botany.)



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FIGURE 17.—Rack to carry 100 tubes. Note polythene above middle rigid sheet, by which the tubes are gripped when being used uncorked. (Photo—courtesy of Journal of Experimental Botany.)



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FIGURE 18.—Clinostat in which 4 racks of tubes are rotated. Driving motor is at right; adjustable balancing arm at left. (Photo—courtesy of Journal of Experimental Botany.)

Variation used by Pegg—Place 2 coleoptile segments in a glass tube 80 × 15 mm. containing 1 ml. of solution to be tested. Seal with a close-fitting plastic or rubber cap. Place the tubes horizontally on a clinostat or circular drum revolving at 2 r.p.m. in total darkness at 25° C. Allow the segments to grow for 24 hr. Prepare 10 tubes for each concentration to be tested and 10 tubes containing distilled water and control segments. Measure responses as described by Wightman.

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 Method submitted by F. Wightman, Department of Biology, Carleton University, Ottawa, Canada.

YOUNG WHEAT COLEOPTILE METHOD

Based on research by S. T. C. Wright.

Description of method—Young, whole coleoptiles are excised from germinating wheat seeds 24 hr. after sowing. The coleoptiles are floated on aqueous solutions of the compounds to be tested and their length is measured before and after treatment. The growth of the treated coleoptiles is compared with that of untreated ones floating on distilled water. Cell elongation using this method is stimulated by gibberellins and cytokinins, but not by auxins.

Apparatus, Chemicals, and Other Materials

- Petri dishes, 10 cm. in diameter
- Constant humidity box in a room with the temperature controlled at 23°-24° C.
- Forceps
- Scalpel
- Binocular dissecting microscope, low power, 10× magnification
- Whatman Seed Testing Papers, 9 cm. in diameter
- Microscope slides
- Eyepiece micrometer, 10 mm. with 100 divisions
- Camel's-hair brushes
- Pieces of filter paper, Whatman No. 1, 5 × 2.5 cm.
- Parafilm

Clinostat designed to hold test tubes rotating at 1 r.p.m. at 25° C. in darkness

Volumetric flasks, 100 ml.

Test tubes, 7.5 × 1.3 cm.

Analytical balance

3-100 mg. of the substance to be evaluated

250 mg. of kinetin or gibberellic acid A₃ for standard treatment

Distilled water

Suggested plant material—Germinating wheat seeds (*Triticum vulgare* Vill. Eclipse variety).

Preparation and selection of plant material—Soak wheat seeds in distilled water for 2 hr. Sow them on moist seed-testing paper in petri dishes, keeping the embryos oriented uppermost. The seeds should be sown at a density of 50 per dish and placed in a constant humidity box (80-85% relative humidity) for 24 hr. at 23° C. in darkness.

Procedure—Using fig. 19 as a guide, excise the coleoptiles with a scalpel under a binocular dissecting microscope. Place the coleoptiles, with their enclosed primary leaves, on filter paper moistened with distilled water until a sufficient number of them have been cut for the experiment. Arrange the coleoptiles in groups of 5 on microscope slides and measure them under the binocular dissecting microscope with a micrometer eyepiece. Carefully transfer each group of 5 coleoptiles with a camel's-hair brush to a strip of filter paper and insert them into a test tube containing 0.6 ml. of the test solution. The purpose of the filter paper is to prevent drying of the coleoptiles on the sides of the tubes. Use 5 replicates of 5 coleoptiles per treatment, including the water controls. Seal the tubes with Parafilm and rotate them in a hori-

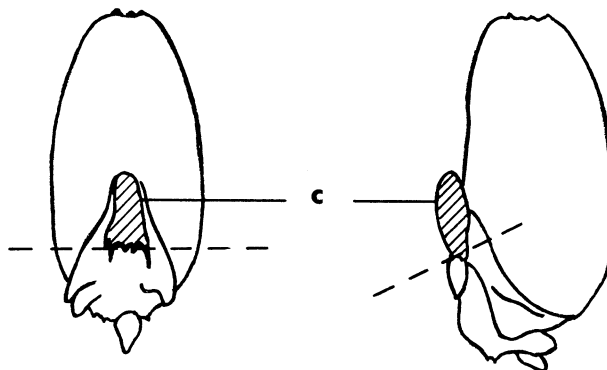


FIGURE 19.—Germinating wheat seed showing coleoptile (c) and plane of incision (broken lines) used to remove coleoptile. (After diagram by S. T. C. Wright.)

zontal position at 1 r.p.m. on a clinostat in darkness at 25° C. for 24 hr. Remove the coleoptiles from the tubes and measure them again.

Method of taking results—Calculate for each tube the mean length of the 5 coleoptiles before and after treatment and from these 2 figures obtain the percentage increase in the mean length of the 5 coleoptiles. Determine the average of these percentages for the 5 replicate tubes. Compare the average growth stimulation produced by the substance under evaluation with the average growth of the coleoptiles in the test tubes containing distilled water.

Suggested standard for comparison—Gibberellic acid at a concentration of 10^{-5} M (i.e., 3.46 mg./liter) and kinetin at a concentration of 10^{-4} M (i.e., 21.5 mg./liter). Dosage response curves may be prepared using the following concentrations: for gibberellic acid— 10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} , 10^{-5} , 10^{-4} M; for kinetin— 10^{-8} , 10^{-7} , 10^{-6} , 10^{-5} , 10^{-4} , and 3.16×10^{-4} M.

Reference

Wright, S. T. C. A Sequential Growth Response to Gibberellic Acid, Kinetin and Indolyl-3-Acetic Acid in the Wheat Coleoptile (*Triticum vulgare*). *Nature* 190: 699-700. 1961.

CELL ENLARGEMENT

CITRUS PETAL METHOD

Based on research by E. E. Goldschmidt and S. P. Monselise.

Description of method—The degree of curvature of detached citrus petals is used to measure the indole-3-acetic acid content of the agar medium in which the petals are implanted. This method can also be used to detect and measure other synthetically prepared growth-regulating substances and has been used to assay extracts of citrus tissue.

Apparatus, Chemicals, and Other Materials

Petri dishes, 5 cm. in diameter
Glass bell jars or other watertight containers to enclose dishes and maintain humid atmosphere
Constant temperature chamber, about 20° C.
Razor blade
Protractor or other device to measure angle of curvature
Analytical balance
0.2–1.0 g. of indole-3-acetic acid for standard treatment
Agar
Sucrose

Suggested plant material—Citrus flowers, collected just before the petals open. Different species and varieties of the *Citrus* genus may be used.

Preparation and selection of plant material—After the agar preparations and equipment are ready to use, collect citrus flowers at the stage at which the petals would normally open within a few hours if the buds were left on the tree. This stage of development is indicated by the

separation of one of the petals from the others (fig. 20, b).

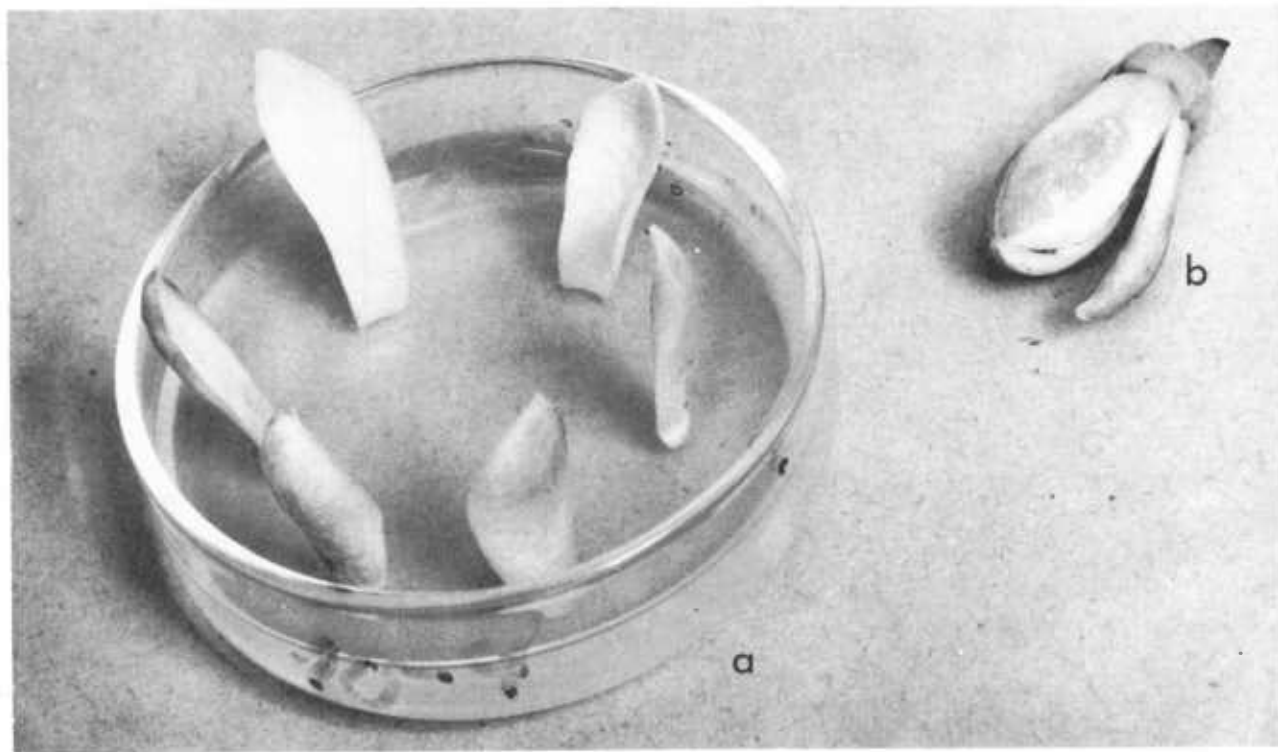
Sever the petals of several flowers at their juncture with the floral axis. Make a random sample of the petals collected by mixing them thoroughly.

Procedure—Prepare 1% agar. Add an amount of sucrose equal to 3% of the weight of the agar mixture. Place 5.0–6.0 ml. of this agar mixture in each of 5 petri dishes. Use this as the control medium to measure variation in the behavior of untreated petals. Prepare appropriate concentrations of the compound or plant extract to be tested by dissolving the required amount of the compound or extract in measured amounts of the agar-sucrose mixture. Use 5 replicate dishes for each concentration.

An alternative method is to pour 1 ml. of solution containing the compound or extract to be tested over the surface of a petri dish after the agar-sucrose mixture has solidified in the dish. Rotate the dish so that the added solution wets the entire surface of the agar. Repeat to make 5 replications of each treatment.

Implant 6 petals into the agar in each dish so that their bases are embedded to a depth of about 2 mm. and the petals are held upright (fig. 20, a). Arrange the petals with their ventral side facing the center of the dish so they can curve freely. Place the dishes in a humid atmosphere at 20° C. in either darkness or continuous light for 22–24 hr.

Method of taking results—After 22–24 hr. under the conditions described above, measure the degree of curvature (fig. 21, angle α) with a protractor. The curvature produced is proportional, within limits, to the concentration of the growth-regulating compound in the agar.



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FIGURE 20.—Arrangement of citrus blossom petals implanted in agar in a petri dish (a) and citrus blossom (b) at stage of development suitable for collection of petals.

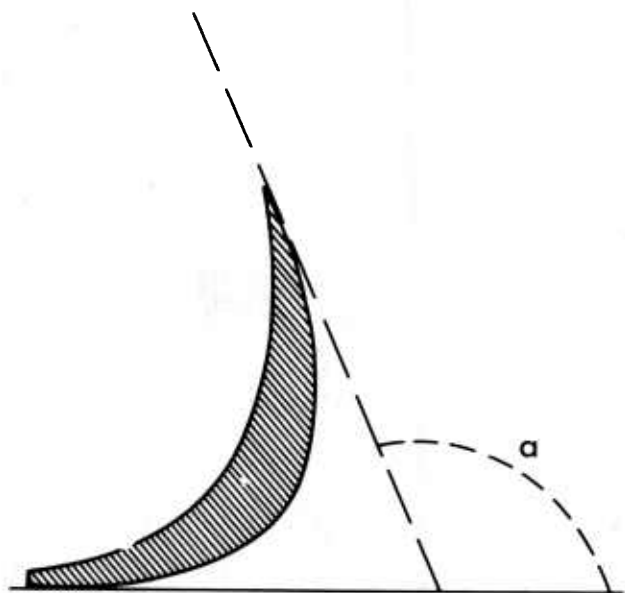


FIGURE 21.—Angle (a) used to measure curvature response of citrus petals to growth substances.

Suggested standard for comparison—Solutions of indole-3-acetic acid at a concentration of 0.02–200 p.p.m. used at the rate of 1 ml. per dish. If the test compound is mixed with the agar during preparation of the agar-sucrose medium, use equivalent amounts of the test compound.

Reference

Goldschmidt, E. E., and Monselise, S. P., Citrus Petal Bioassay Based on the Indolyl-3-acetic Acid Effect on Flower Opening. *Nature* 212: 1064–1065. 1966.

LEAF DISK ENLARGEMENT METHOD

Based on research by C. O. Miller.

Description of method—Etiolated disks from bean leaves are placed on filter paper moistened with nutrient solution in which the compound to be tested is dissolved. Increase in the diameter of the disks when these are kept in darkness is used as a basis for measurement of growth-regulating activity. This method is used to detect kinetin and cytokinins.

Apparatus, Chemicals, and Other Materials

Coarse sand
 Dark room with temperature controlled at about 25° C.
 Cork borer, 5 mm. in diameter
 Analytical balance
 Green safelight of low intensity
 Petri dishes
 Filter paper disks, Whatman No. 1, 9 cm.
 Dissecting microscope with eyepiece micrometer
 KNO₃
 250 mg. of kinetin or gibberellic acid A₃ for standard treatment
 D-glucose
 Distilled water

Suggested plant material—Dwarf stringless greenpod beans (*Phaseolus vulgaris* L.).

Preparation and selection of plant material—Plant the seeds in coarse sand and germinate them in a dark room at about 25° C. for 7–9 days until the primary leaves are about 2 cm. sq. in area. Cut disks 5 mm. in diameter with a cork borer from the 2 primary leaves of the etiolated plants. Cut 2 disks from each leaf, one from the basal section on each side of the mid-vein. A section of a main lateral vein approximately bisects each disk. Perform all manipulations involving the disks in dim green light.

Procedure—Make a basal nutrient solution that contains 3% D-glucose by weight and 0.08 M KNO₃. The pH of this solution should be ap-

proximately 5.6. Add 5 ml. of the test solution to a petri dish containing 3 disks of the filter paper. Place 10 of the leaf disks, lower epidermis up, on the pad of filter paper, wetted with the test solution. Maintain the petri dishes containing the leaf disks in a dark cabinet at 24°–26° C.

Marked disk expansion is induced by exposing leaf disks on nutrient solution only to 200 foot-candles of light from an incandescent filament for 15 minutes before placing the disks in a dark cabinet. Short exposures to red light are also very effective; such exposures are reversed by far red. Co(NO₃)₂ • 6H₂O will also accelerate leaf disk expansion when the salt is added to the nutrient solution in Co⁺⁺ concentrations of 2–5 p.p.m. (about 25 mg./liter of Co(NO₃)₂ • 6H₂O).

Method of taking results—Three days after beginning the experiment, measure the diameter of each disk. Measure at right angles to the heavy veins that bisect each disk. Compare the increase in diameter of the disks on the paper impregnated with nutrient containing the test solution with disks on paper impregnated with the nutrient solution only. Increase in diameter of disks indicates amount of growth regulator activity.

Suggested standard for comparison—Kinetin and gibberellic acid (at about 5 mg./liter).

References

- Miller, C. O. Promoting Effect of Cobaltous and Nickelous Ions on Expansion of Etiolated Bean Leaf Disks. Arch. Biochem. and Biophys. 32: 216–218. 1951.
 Miller, C. O. Similarity of Some Kinetin and Red Light Effects. Plant Physiol. 31: 318–319. 1956.

CHLOROPHYLL RETENTION**BARLEY LEAF METHOD**

Based on research by H. Kende.

Description of method—Segments from leaf blades of young barley plants are conditioned and then floated on the solution being tested for the presence of substances with kinetin-like activity. The segments are then extracted with ethanol and the chlorophyll lost during the storage period is measured in terms of optical density. Retention of chlorophyll by the leaf segments compared with loss of chlorophyll from leaf segments floated on water represents the kinetin activity in the sample of the solution being tested.

Apparatus, Chemicals, and Other Materials

Razor blade
 Beakers, 500 ml.
 Screw-cap vials
 Densimeter
 Analytical balance
 250 mg. of kinetin for standard treatment
 Penicillin G
 80% ethanol

Suggested plant material—Barley plants (*Hordeum distichon* L.) about 2 wk. old.

Preparation and selection of plant material—Germinate barley seeds in soil at 20° C. under continuous illumination from a fluorescent light for

13 days. Select plants for uniformity and cut a segment from each first leaf. Take a 1-cm. segment from the leaf, severing the blade so the distal end of the segment is about 3 cm. from the tip of the leaf. Age the segments for 24 hr. while floating them on distilled water at 25° C. in darkness.

Procedure—Remove the leaf segments from the aging bath, blot them, and transfer 4 segments to each of 3 vials, each vial containing 1 ml. of test solution and 250 units of penicillin G.

Method of taking results—At the end of 48 hr. of incubation at 25° C. in darkness, remove each group of 4 segments from each vial separately.

Extract each group with 5 ml. of 80% ethanol. Measure the optical density of each extract using a wavelength of 665 millimicrons. Compare the optical densities obtained with those of a standard curve made by subjecting barley leaves under comparable conditions to amounts of kinetin varying from 0.003–3.0 micrograms/ml. Express the results in terms of equivalent amounts of kinetin.

Suggested standard for comparison—Kinetin in concentrations ranging from 0.003 to 3.0 micrograms/ml.

Reference

Kende, H. Preservation of Chlorophyll in Leaf Sections by Substances Obtained from Root Exudate. *Science* 145: 1066–1067. 1964.

COCKLEBUR LEAF DISK METHOD

Based on research by Daphne J. Osborne and D. R. McCalla.

Description of method—Disks cut from leaves at a specific stage of senescence are placed on filter paper. Known amounts of kinetin are added to the filter paper as standards. Comparable disks are placed on filter paper moistened with the solution being tested or on a moistened segment of chromatogram containing separated components of an extract. The presence of kinetin-like compounds is indicated by a suppression of yellowing due to the retardation of chlorophyll degradation in the leaf disks. The quantity of these compounds present is estimated by comparing the loss of chlorophyll in disks in contact with the unknown sample with the loss in disks in contact with known amounts of kinetin. The test is quantitative over the range from 0.05–5.0 micrograms of kinetin. It

is especially useful in testing zones cut from paper chromatograms and may also be used for materials in solution.

Apparatus, Chemicals, and Other Materials

Temperature-controlled room
Means of supplementing daylight with incandescent light to obtain a 20-hr. photoperiod
Plexiglas boxes for storage of leaves
Means of supplying leaves with light from incandescent lamp (50 foot-candles)
Filter paper disks, Whatman No. 1, 4.25 cm. in diameter
Petri dishes, 5 cm. in diameter
Cork borer, 12 mm. in diameter
Graduate cylinder, 100 ml.
Large petri dish in which 5-cm. petri dishes can be stored
Enamel trays in which large petri dishes can be stacked
Aluminum foil
Dark area at 24° C.
Graduated centrifuge tubes
Water bath
Beckman spectrophotometer, Model B
250 mg. of kinetin for standard treatment
Ethyl acetate
Acetic acid
80% ethanol

Suggested plant material—Cocklebur (*Xanthium pennsylvanicum* Wall.). Other kinds of senescent leaf tissues may be used satisfactorily in this method as an assay for cytokinins, for example, radish (*Raphanus sativus* L.), wheat (*Triticum sativum* Lam.), and barley (*Hordeum vulgare* L.), but these are less sensitive than cocklebur.

Preparation of plant material—Grow the cocklebur plants from seed in the greenhouse at temperatures from 20°–30° C. Supplement daylight with incandescent light to give a photoperiod of 20 hr. in order to maintain plants in the vegetative condition. After the plants have produced 10 leaves, remove the fifth fully expanded leaf (counting back from the apex) leaving the petiole attached. Store the detached leaves with only their petioles under water at low light intensity (50 foot-candles from an incandescent lamp). Enclose the leaves in Plexiglas boxes during this storage period and maintain a temperature of 20° C. After 3 days under these conditions, during which the first stages of senescence take place, select for experimental use those leaves whose blades are uniformly pale green.

Procedure—Prepare kinetin standards at a concentration of 0.063–16 mg./liter. Apply 0.5 ml. of each of these concentrations to the cor-

responding disks of filter paper in the petri dishes. Prepare control filter papers in similar dishes and add 0.5 ml. of distilled water to each dish.

To test chromatographed plant extracts, segment the chromatogram into strips not less than 12 mm. in the narrow dimension. Cut the long dimension of the strips to 4.25 cm. and place each chromatographed segment in a petri dish. Moisten each segment of the chromatogram by adding 0.5 ml. of distilled water to each dish. To avoid toxic materials, make sure that chromatographic paper used for separating components of plant extracts is washed thoroughly before the separation. Use ethyl acetate, acetic acid, water (10:5:2, v/v) for this preliminary wash and glass-distilled water for the second wash.

Cut uniform disks 12 mm. in diameter from interveinal blade tissue of the detached leaves previously aged for 3 days as described above. (Sixty disks may be obtained from each suitable leaf.) Place 4 of these disks, or a larger number of smaller disks, in each petri dish so they are resting with their abaxial surfaces in contact with the filter paper disks or the chromatogram segments.

Extract duplicate sets of 4 (or more) leaf disks from those originally obtained by boiling them in 80% ethanol. Retain the extract in the dark for use in determining the original chlorophyll content of the disk.

Place each 5-cm. petri dish with its enclosed papers and disks on 2 layers of damp filter paper in a larger petri dish. Close the larger dishes and stack them in enamel trays lined with damp filter paper and cover the entire assembly with aluminum foil. Maintain them in darkness for 48 hr. at 24° C. At the end of this period, examine the leaf disks. The control leaf disks should be yellow or yellow green, and the chlorophyll almost completely degraded; disks in the higher concentrations of kinetin should still be green. If this difference does not exist, prolong the storage period.

Drop each group of disks from the various dishes into corresponding graduated centrifuge tubes with each tube containing a 5–6 ml. of hot 80% ethanol. Boil the ethanol in each tube gently on a water bath until the chlorophyll has been extracted. Cool the tubes and adjust the volume to 10 ml., using 80% ethanol. Measure the optical density of each solution with a Beckman spectrophotometer Model B (or other suitable instrument) at 665 millimicrons, the absorption maximum in the red region for chlorophyll *a*.

Method of taking results—Compare graphically the loss of chlorophyll in leaf disks exposed to

various concentrations of kinetin with the loss of chlorophyll from leaf disks exposed to the unknown components of an extract or paper chromatogram. The retention of chlorophyll bears a linear relation to the logarithm of the kinetin concentration over a range of approximately 0.05–5.0 micrograms (0.1–10 mg./liter).

Indole-3-acetic acid and other synthetic regulating compounds tested so far have not retarded chlorophyll loss in this test.

High concentrations of gibberellin A₃ show slight activity.

Suggested standard for comparison—Kinetin in concentrations ranging from 0.063–16 mg./liter.

Reference

Osborne, Daphne J., and McCalla, D. R. Rapid Bioassay for Kinetin and Kinins Using Senescing Leaf Tissue. *Plant Physiol.* 36: 219–221. 1961.

DOCK LEAF DISK METHOD

Based on research by Pamela Whyte and L. C. Luckwill; R. A. Fletcher and Daphne J. Osborne.

Description of method—To test for the presence of gibberellin-like hormones, disks cut from dock leaves are placed on filter paper moistened with a solution of the compound to be tested. Comparable disks are placed on paper moistened with distilled water and all the disks are kept in darkness for 4 or 5 days. The chlorophyll content of the treated disks is then compared with that of the control disks. The amount of chlorophyll retained by the treated disks is proportional, within certain limits, to the logarithm of the amount of gibberellin-like substance present. This test is extremely sensitive to small amounts of gibberellic acid A₃.

Apparatus, Chemicals, and Other Materials

Temperature-controlled room, 25° C.

Clay pots

Greenhouse space

Filter paper

Small glass dishes

Petri dishes

Spectrophotometer

Cork borers, 2.5 cm. and 7 mm. in diameter

Pipettes, 0.1-ml. graduations

100–500 mg. of gibberellic acid A₃ for standard treatment

Methanol or acetone

Distilled water

Suggested plant material—Broadleaf dock (*Rumex obtusifolius* L.).

Preparation and selection of plant material—Propagate, clonally, dock plants in soil contained in clay pots under greenhouse conditions. Select the oldest leaves that are still uniformly green. Detach these leaves and immerse their petioles in water for 24 hr. in darkness.

Procedure—Cut disks 2.5 cm. in diameter from filter paper. Place each of these in a small glass dish. Moisten each filter paper disk with 0.3 ml. of the test solution. Cut 4 leaf disks, 7 mm. in diameter, each from a separate leaf. Place these 4 leaf disks on one filter paper disk, the abaxial surfaces down. Repeat the procedure, using separate dishes and distilled water rather than the test solution to moisten the filter paper disks. Make 4 replications of this arrangement. Place the small glass dishes containing the filter paper and leaf disks in petri dishes lined with moistened filter paper and incubate at 25° C. in darkness. Place the small dishes containing the treated disks and those containing the control disks in separate petri dishes.

Method of taking results—When the chlorophyll has almost disappeared from the control leaf disks—usually after 4 or 5 days—place the 4 leaf disks of each replicate separately in 6 ml. of methanol or acetone. Extract the chlorophyll at room temperature overnight and determine the optical density of the resulting solutions at 665 millimicrons (chlorophyll *a*) with the spectrophotometer. Express the chlorophyll content of the treated leaf disks as percentage of that of the control.

Suggested standard for comparison—Gibberellic acid A₃ 0.00001–0.1 microgram/ml.

Variation used by Fletcher and Osborne—Grow dandelion (*Taraxacum*) plants under greenhouse conditions or use plants growing outdoors. Detach mature, fully expanded leaves that are uniformly pale green. If young leaves are used, place them under a bell jar with petioles in water to “age.” Keep the leaves in this condition until their glossy green color fades to a uniformly pale green. Cut interveinal portions of convenient diameter from the leaves with a cork borer. Extract duplicate sets of disks at once with hot 80% ethanol. Store this extract in darkness for subsequent determination of the original chlorophyll content. Place other matched sets of disks in petri dishes on filter paper moistened with either water or the test solution. Also use a graduated series of

gibberellic acid concentrations for comparison. Do not use too much solution to moisten the filter paper; e.g., 0.8 ml. of solution per 7-cm. diameter filter paper is satisfactory. Place the abaxial surface of the leaf disks in contact with the moist filter paper. Line the covers of the petri dishes with moistened filter paper. Place the closed petri dishes containing the leaf disks in a plastic box with high humidity and store in darkness at 25° C. for about 4 days.

Place each set of disks separately into test tubes containing hot 80% ethanol and boil gently. Stopper the tubes and transfer them to an incubator maintained at 37° C. overnight to complete the extraction of chlorophyll. Less time is required by boiling the disks in the ethanol until all the chlorophyll has been extracted. Bring the chlorophyll extracts to a uniform volume with 80% ethanol and determine the absorbency of each solution and that of the initial controls at 665 millimicrons with a suitable colorimeter.

References

- Whyte, Pamela, and Luckwill, L. C. A Sensitive Bioassay for Gibberellins Based on Retardation of Leaf Senescence in *Rumex obtusifolius* (L.). *Nature* 210: 1360. 1966.
Fletcher, R. A., and Osborne, Daphne J. A Simple Bioassay for Gibberellic Acid. *Nature* 211: 743–744. 1966.

WHEAT LEAF SENESCENCE METHOD

Based on research by S. T. C. Wright.

Description of method—Detached wheat leaves age and turn yellow more quickly when floated on water than when floated on solutions of cytokinins. The degree of senescence is determined by extracting the leaves with ethanol and estimating the chlorophyll content spectrophotometrically 4 days after detachment.

Apparatus, Chemicals, and Other Materials

- Forceps
- Vermiculite, No. 4
- Plastic seed trays, 36 cm. long × 23 cm. wide × 5 cm. deep
- Growth room with a 15-hr. photoperiod (500 lumens/sq. ft. from fluorescent tubes giving “natural daylight”) at 23° C.
- Incubator at 25° C.
- Cm. rule
- Scissors
- Cutting board
- Analytical balance
- Volumetric flasks, 100 ml.

Petri dishes, 10 cm. in diameter
 Pyrex test tubes, 10 × 1.5 cm.
 Pipette, 10 ml.
 Test tube wire baskets, divided
 Thermometer, 0°–100° C.
 Suitable vessel for water bath
 Glass marbles
 Stop watch
 Bunsen burner
 Black cloth
 Spectrophotometer or colorimeter capable of measuring light absorption at a wavelength of 665 millimicrons
 80% ethanol
 250 mg. kinetin for standard treatment
 3–100 mg. of compound to be evaluated
 Distilled water

Suggested plant material—Seedlings of wheat (*Triticum vulgare* Vill.) Eclipse variety.

Preparation and selection of plant material—Soak wheat seeds in distilled water for 2 hours. Using forceps, sow the seeds in moist vermiculite in the seed trays with the embryos oriented uppermost and with about 1.5 cm. between the seeds and rows. Cover the seeds with a thin layer of vermiculite and compress the surface with a firming board. Place the trays in the growth room for 7 days.

Procedure—Excise the shoots (approximately 11 cm. long) with scissors, cutting just above the soil level, and line them up on a cutting board with their tips against a wooden guide. Sever the shoots 7.5 cm. from the tips. This severed portion of the shoot comprises mainly the first leaf but clasped within it are portions of other leaves. Use only the first leaf; remove and discard the others. Weigh the leaves in batches

of 3 to the nearest mg. and float each batch, with their adaxial surfaces uppermost, on 10 ml. of test solution contained in a petri dish. Use 3 replicate dishes for each treatment and 3 dishes containing distilled water as controls. Extract 3 sets of leaves from untreated plants immediately in boiling 80% ethanol (as described below) and store the extracts in darkness at 2° C. for determining the original chlorophyll content of the leaves. Maintain the petri dishes at 25° C. for 4 days in darkness.

Method of taking results—At the end of the 4-day treatment period, transfer the leaves from each petri dish to a test tube and pipette 10 ml. of 80% ethanol into the tube. Stopper the test tubes with glass marbles to prevent evaporation. Place the tubes in a vertical position in a test tube wire basket. Immerse the basket containing the tubes in a water bath. Bring the water bath temperature up to 80° C. and hold at this temperature for 10 minutes. Remove the tubes containing the boiling ethanol from the water bath. Since chlorophyll extracts fade quickly in bright sunlight, cover the tubes with a black cloth while they are cooling. Measure the optical density of each solution against 80% ethanol (using a 0.5-cm. cell) in a spectrophotometer at 665 millimicrons, the absorption maximum in red light of chlorophyll *a*. Express the results as optical density/mg. of leaf.

Suggested standard for comparison— 10^{-4} M solution of kinetin (21.5 mg./liter). A dosage response curve can be prepared by using the following concentrations of kinetin: 10^{-8} , 10^{-7} , 10^{-6} , 10^{-5} , 10^{-4} , and 3.16×10^{-4} M.

Reference

Method submitted by S. T. C. Wright, Plant Growth Substance and Systemic Fungicide Unit, Agricultural Research Council, Wye College, Near Ashford, Kent, England.

EFFECT OF ONE COMPOUND ON THE ABSORPTION AND TRANSLOCATION OF ANOTHER

BEAN STEM CURVATURE METHOD

Based on research by A. S. Crafts; J. W. Mitchell, W. M. Dugger, Jr., and H. G. Gauch.

Description of method—The magnitude of stem curvature induced by a known amount of a growth-modifying substance is compared with that induced by the same amount of the substance plus an adjuvant.

Apparatus, Chemicals, and Other Materials

See p. 11.

Compounds to be tested as adjuvants are also required.

500 mg. of ammonium 2,4-dichlorophenoxyacetate for standard treatment

Suggested plant material—See p. 11.

Preparation and selection of plant material—See p. 11.

Procedure—Using the “Bean stem curvature method,” p. 12, determine the amount of ammonium 2,4-dichlorophenoxyacetate or other water-soluble salts of the acid required to induce a stem curvature of about 5° – 10° in $2\frac{1}{2}$ –3 hr. In these preliminary tests, dissolve the salt directly in distilled water and use a concentration range that includes 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 $\mu\text{g.}$ of the salt per 0.02 ml. of solution.

After determining the threshold concentration, prepare 500 ml. of this concentration. Divide it into the required number of 50-ml. portions. Reserve one portion to be applied as a control, and mix each adjuvant to be tested with a 50-ml. portion. Apply to test plants as described on p. 12.

Method of taking results—Determine the effect of the adjuvants on the magnitude of stem curvature induced in $2\frac{1}{2}$ –3 hr. by comparing the average curvature developed by plants treated with the salt alone with that developed by plants treated with the salt plus the adjuvant.

Suggested standard for comparison—0.8, 0.9, or 1.0 $\mu\text{g.}$ of ammonium 2,4-dichlorophenoxyacetate or other water-soluble salt applied in distilled water alone.

References

- Crafts, A. S. Herbicides, Their Absorption and Translocation. *Agr. and Food Chem.* 1: 51–55. 1953.
 Mitchell, J. W., Dugger, W. M., Jr., and Gauch, H. G. Increased Translocation of Plant Growth-Modifying Substances Due to Application of Boron. *Science* 118: 354–355. 1953.

ENZYME PRODUCTION

BARLEY ENDOSPERM METHOD

Based on research by J. E. Varner and G. R. Chandra; R. L. Jones and J. E. Varner.

Description of method—Endosperm of barley seeds releases alpha amylase and other enzymes in the presence of gibberellic acid A_3 and some gibberellin-like substances. The amount of enzymes released is measured in terms of amyolytic activity, which is proportional, within limits, to the logarithm of the concentration of gibberellic acid A_3 or gibberellin-like substances present.

Apparatus, Chemicals, and Other Materials

Razor blade
 Petri dishes, 10-cm.
 Sand
 Erlenmeyer flasks, 25-ml.
 Pipettes, 1- and 10-ml. volume
 Autoclave
 Shaker apparatus
 Centrifuge
 Centrifuge tubes, 100×7.5 mm.
 Spectrophotometer
 Sodium hypochlorite, 1% (commercial bleach)
 500 ml. of acetate buffer (pH 4.8)
 10 g. of calcium chloride
 1 g. of chloramphenicol
 5 g. of potato starch (nonsolubilized)
 10 g. of potassium phosphate
 5 g. of potassium iodide
 1 oz. of hydrochloric acid

5–10 g. of iodine
 1 p.p.m. of gibberellic acid A_3 solution for standard treatment
 Sterile water

Suggested plant material—Seeds of any variety of barley (*Hordeum vulgare*) that contain a relatively small amount of endogenous gibberellin are suitable. One such cultivar is ‘Himalaya.’

Preparation of plant material—Cut 30–40 barley seeds transversely and discard the portion of each seed that contains the embryo. Sterilize the halves containing endosperm by soaking them in 1% sodium hypochlorite for 20 minutes. Wash the seed halves three times with sterile water. Ignite sand to sterilize it and place 100 g. of it in a petri dish. Moisten the sand with 20 ml. of sterile water. Place the sterilized pieces of seeds on the sand and cover the petri dish. Allow the seed pieces to imbibe water for 3 days at room temperature. Use the remaining seed pieces in lots of 10 in a comparable way as replications.

Procedure—Transfer aseptically 10 seed pieces to an Erlenmeyer flask containing 2 micromoles of the acetate buffer and 20 micromoles of calcium chloride. Autoclave the calcium chloride-buffer mixture. Add enough of the compound to be tested or of the extract dissolved in 2 ml. of sterile water to give a suitable amount of amyolytic activity (determined with exploratory tests). Add 20 micrograms of chloramphenicol to each flask. Close the flasks and incubate them

for 24 hr. at 25° C. with continuous shaking at 40 oscillations/minute. Decant the liquid from each flask into a centrifuge tube and wash the seeds with 3 ml. of sterile water. Combine the wash water with the decantate. Centrifuge the tubes for 10 minutes at 2,000 times gravity.

Method of taking results—Transfer a suitable volume of the centrifuged supernatant liquid (0.02–0.2 ml.) from each tube to a separate flask, adding enough sterile water to make a total volume of 1 ml./flask. Add 1 ml. of starch substrate prepared by boiling a mixture of 150 mg. of the potato starch, 600 mg. of KH_2PO_4 , and 2 mg. of calcium chloride in 100 ml. of distilled water for one minute. Centrifuge for 10 minutes at 3,000 times gravity. Decant the supernatant liquid for use in the assay. Allow the enzymatic digestion of starch to proceed for 10 minutes or for the required time previously determined with exploratory tests. Stop the reaction by adding 1 ml. of iodine reagent prepared by mixing 6 mg. of potassium iodide and 600 mg. of iodine in 100 ml. of water and adding 1 ml. of this stock solution to 0.05 N hydrochloric acid to give a final volume of 100 ml. Add 5.0 ml. of distilled water to each of the final mixtures. Determine the optical density of each mixture at 620 millimicrons. On the basis of ex-

ploratory tests, adjust the enzyme volume and time of hydrolysis so that the optical density of the starch-iodine complex is about half that of the zero time control. Convert the optical density values to micrograms of alpha-amylase using a factor obtained for each starch sample by standardization with a pure amylase preparation.

Calculate the results arbitrarily as units of alpha-amylase:

$$\frac{\Delta OD \times V}{T \times V'} = 1 \text{ unit of alpha-amylase}$$

ΔOD = absorbency of zero time control minus absorbency of sample after hydrolysis for time (T) measured in minutes

V = total volume of enzyme solution expressed as ml.

V' = volume of enzyme solution taken for assay expressed as ml.

Suggested standard for comparison—Gibberellic acid A_3 , 0.1–1.0 microgram/ml.

References

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Varner, J. E., and Chandra, G. R. Hormonal Control of Enzyme Synthesis in Barley Endosperm. *Natl. Acad. Sci. Proc.* 52: 100–106. 1964.

EXUDATION OF REGULATORS FROM ROOTS

BEAN METHOD

Based on research by W. H. Preston, Jr., J. W. Mitchell, and W. Reeve; P. J. Linder, J. W. Mitchell, and Greta D. Freeman.

Description of method—Two young plants, one treated by placing a compound on its stem or leaves, are grown in soil contained in a pot. Formative or other effects that become apparent as the untreated plants develop indicate transfer of the compound from one plant to the other through the root systems.

Apparatus, Chemicals, and Other Materials

Clay pots, 8, 10, or 13 cm.
Composted soil
Greenhouse space maintained at 21°–29° C., slightly cooler at night
Shell vials, about 10-ml. capacity
Analytical balance
Small glass or wooden rods used as applicators, 1- to 2-mm. diameter, 5–10 cm. long
250–500 mg. of each compound to be evaluated

Alpha-methoxyphenylacetic acid for standard treatment

About 50 ml. of Tween 20

About 50 g. of lanolin

Beakers, 200–400 ml.

Source of compressed air

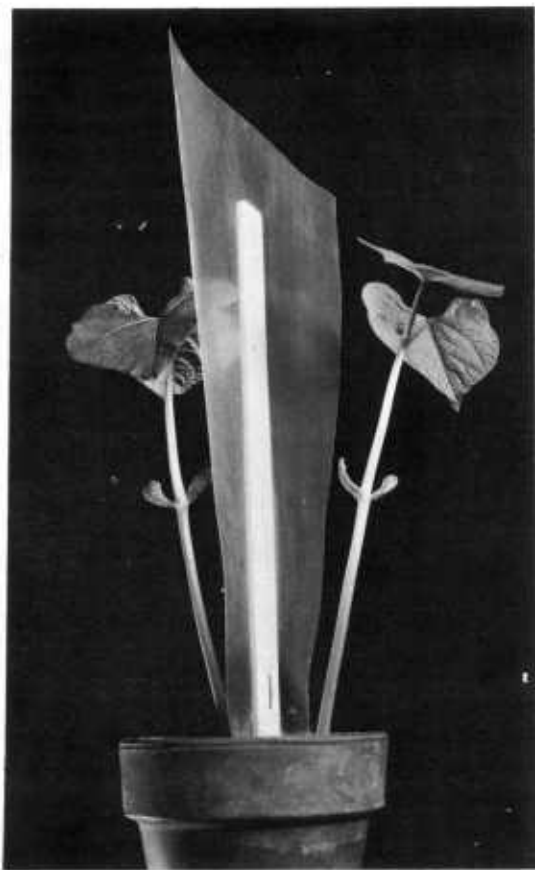
Suggested plant material—Young herbaceous dicotyledonous plants known to respond morphologically to the compounds to be tested—snap bean (*Phaseolus vulgaris* L.), cucumber (*Cucumis sativus* L.), tomato (*Lycopersicon esculentum* Mill.), squash (*Cucurbita* spp.), sunflower (*Helianthus annuus* L.).

Preparation and selection of plant material

Plant 3 or 4 seeds per pot in composted soil if a single species is used. If 2 species are used, plant 3 or 4 seeds of each per pot. After germination, allow the plants to grow until the first leaf formed above the cotyledons is partially expanded. While the next leaves are still tightly folded in the terminal buds, select in each pot 2 vigorously growing plants that are the same species or of 2 different species. Discard all

plants except these 2 by severing their stems near the soil level. Arrange 3-5 pots (each containing a pair of plants) in a row to be used for each compound to be tested. Use a sheet of plastic to prevent the aboveground parts of the treated plant from touching those of the untreated plant (fig. 22).

Procedure—Prepare a 1% lanolin mixture of the compound being tested, as described on pp. 8, 106. With an applicator, apply a portion of the paste about the size of a wheat seed to one plant in each pot in one row. Leave the other plant in each pot untreated. Apply the paste as a band 2-3 mm. wide around the first internode of each plant midway between the first and the second node (or around the upper portion of the hypocotyl if cucumber or sunflower is used). Allow the treated and untreated plants to grow in a greenhouse for a week or two.



BN-28927

FIGURE 22.—Sheet of plastic used to separate aboveground parts of treated plant from aboveground parts of untreated plant.

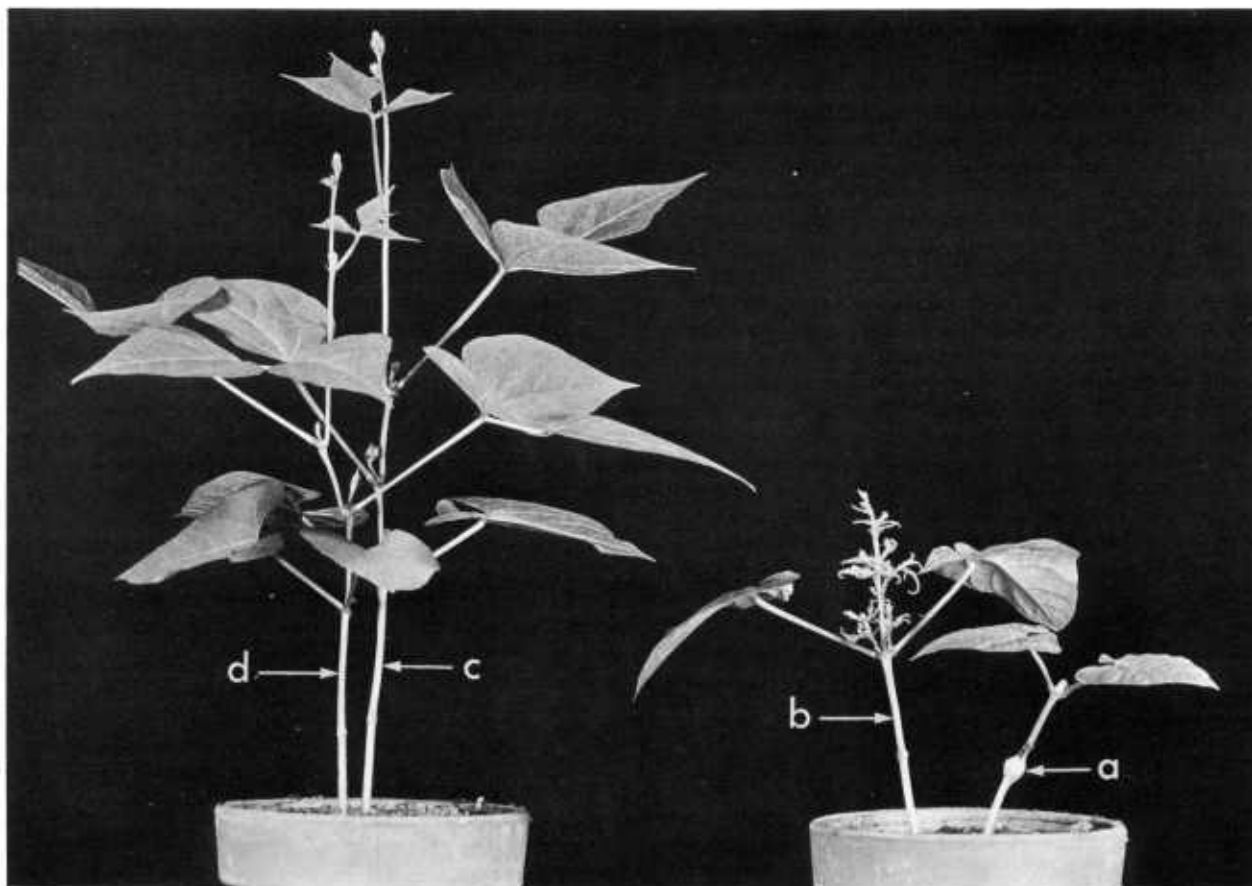
Figure 23 illustrates results of a test with 4 plants grown under similar conditions. Alpha-methoxyphenylacetic acid was applied (arrow) to plant *a* shown at the extreme right. The acid was absorbed by the plant and then translocated to the roots where some of the acid was exuded. Plant *b* growing in the same pot, with its roots near those of the treated plant, then absorbed some of the exuded acid, translocated this to its terminal bud where the regulating substance induced growth of malformed leaves. Untreated plants *c* and *d* are shown for comparison.

For an alternative method, carefully wash the roots of at least 3 of the young plants free of soil. Support the plants with their roots immersed in about 200 ml. of tapwater in the beaker. Aerate the water by passing compressed air as fine bubbles through it. Treat the stems of all the plants as described. Arrange a set of untreated plants in another beaker of water as controls. Allow all the plants to grow for 3 days, replacing any water lost. Then remove and discard all the plants. Introduce an equal number of young untreated plants and allow them to grow for several days with their roots in the continuously aerated water that previously supported growth of the treated and untreated plants.

Method of taking results—In the case of plants with their roots in soil contained in the same pot, observe in the untreated plants any growth modification such as deviation in size or shape of newly expanded leaves (formative effects) (fig. 23, *b*). Make a similar comparison for the alternative test using aerated water. (See "Root exudation method," p. 52.)

Supplementary tests: Determination of the path of movement of the compound—In the case of volatile compounds, there is a possibility that the compound may pass from the treated plant to the untreated one through air. To determine the path of movement, select 5 pots of test plants as previously described with 2 plants of uniform size growing in each. Also select 10 other plants, each growing individually in a pot. Cut out 10 squares (5 cm.) of cardboard and punch 2 holes, 1 cm. in diameter and 15 mm. apart, in each square. Make a narrow slit from the edge of the cardboard to each hole. Using the pots containing 2 plants each, slip the cardboard around the stem of each plant so that the stems protrude through the holes and are thus held about 15 mm. apart. Place the pots containing individual plants in pairs and slip a cardboard piece around the stems of each pair of plants in the same manner.

Apply the compound to be tested to the stem of 1 plant of each pair. Allow the plants to grow



BN-28926

FIGURE 23.—Alpha-methoxyphenylacetic acid applied to stem of plant *a* was absorbed by this plant, then translocated to its roots where some of the acid was exuded. Plant *b*, with its roots near those of plant *a*, absorbed some of the exuded acid, translocated this to its terminal bud where the regulating substance induced growth of malformed leaves. Untreated plants *c* and *d* are shown for comparison.

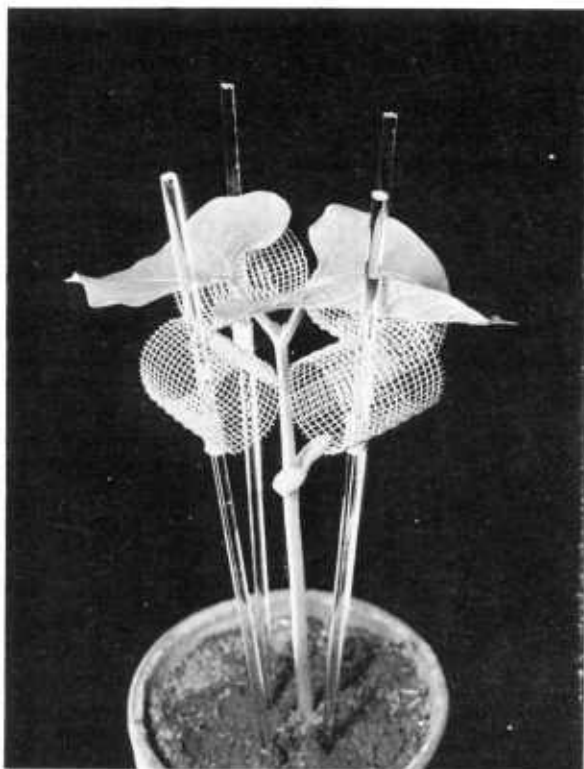
in a greenhouse until the next 2 leaves are partially expanded. Leaf modification in both plants with their roots in separate pots indicates that the compound moved through the air. Modification of leaf shape in both plants growing in soil contained in the same pot, but not in the untreated plants of pairs growing with their roots in separate pots, indicates that the compound passed from one plant to the other through their root systems.

Another test to determine whether the chemical moves from the lanolin mixture through the air in sufficient amounts to affect a plant can be made as shown in fig. 24. Lanolin mixture is applied to each of 4 glass rods that are placed near the test plant. The amount used for each rod and method of application are the same as those described on p. 50. Cylinders of screen wire, used as illustrated (fig. 24), keep the mixture in the treated area on each rod from touch-

ing the plant. Modification of shape or formative effects in new leaves indicate that a volatile growth regulator has moved from the glass rods to the plants in amounts that induce the response.

Still another method of measuring response is as follows: estimate the amount of the compound that exudes from the roots of the plant to which the chemical is applied by comparing the suppression of growth in trifoliolate leaves of a nearby plant with reduction in trifoliolate leaves of other plants treated directly with various amounts of the acid. Make these measurements by determining the fresh weight of growth above the primary leaf node.

Suggested standard for comparison—Alpha-methoxyphenylacetic acid, 1% lanolin-Tween 20 paste.



BN-28951

FIGURE 24.—Testing volatility of compound mixed with lanolin by applying mixture to glass rods placed near test plant and then noting growth responses that develop. Screen wire prevents mixture from touching the plant.

References

- Linder, P. J., Mitchell, J. W., and Freeman, Greta D. Persistence and Translocation of Exogenous Regulating Compounds That Exude from Roots. *Agr. and Food Chem.* 12: 437-438. 1964.
- Preston, W. H., Jr., Mitchell, J. W., and Reeve, W. Movement of Alpha-methoxyphenylacetic Acid from One Plant to Another Through Their Root Systems. *Science* 119: 437-438. 1954.

ROOT EXUDATION METHOD

Based on research by J. W. Mitchell, P. J. Linder, and Melba B. Robinson.

Description of method—The ability of plants to absorb a regulating compound into their leaves, translocate it downward, and exude it from their roots into the surrounding medium is measured with the use of radioactivity or by means of a bioassay.

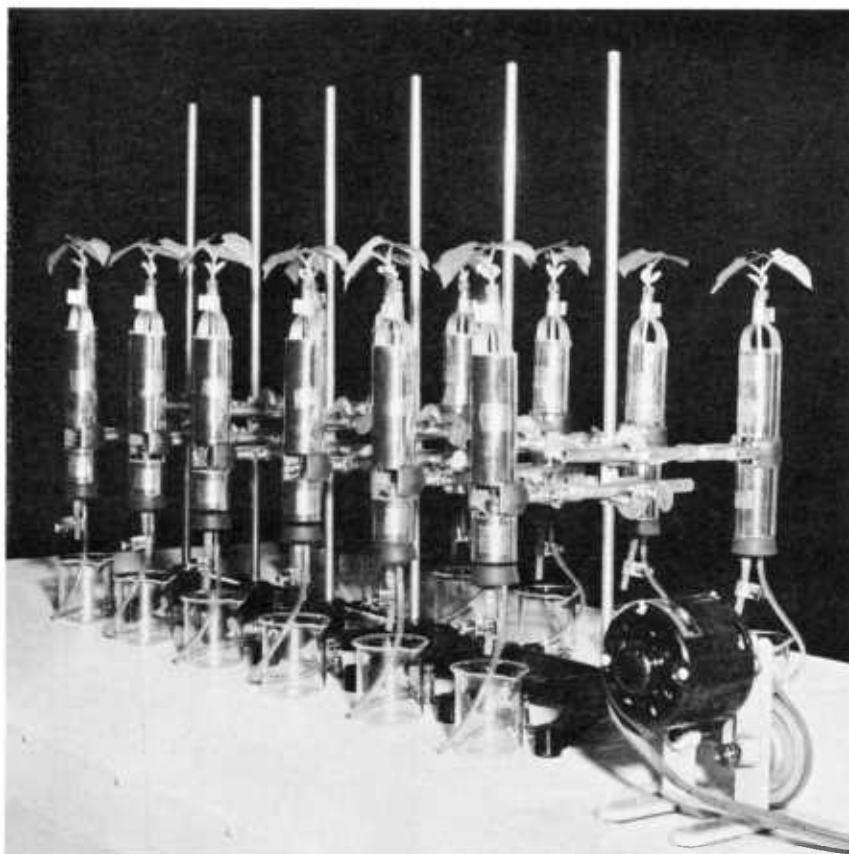
Apparatus, Chemicals, and Other Materials

- Clay pots, 8-10 cm.
- Composted soil
- Razor blade
- Glass tubes, 32 mm. inside diameter, 15-cm. long, to be used as root chambers
- Rubber stoppers, 2-hole, No. 12, 1 for each root chamber
- Soft glass tubing to fit the holes of the rubber stoppers
- Glass tubing, about 5 mm. outside diameter
- Plastic tubing, approximately 4 mm. and 5 mm. inside diameter
- Rubber tubing, 3 mm. inside diameter
- Brass needle valves, 1 for each root chamber
- Pinch clamps, 1 for each root chamber
- Aluminum foil
- Cellophane tape
- Ring stands, 1 for each pair of root chambers
- Sheet metal for constructing plant supports
- Aluminum paint
- Beakers, 100-ml., 1 for each root chamber
- Glass rod, 4 mm. inside diameter, 15 cm. long
- Small aquarium pump
- Burette clamps, 1 for each root chamber
- Analytical balance
- Geiger counter
- 2-10 mg. of the stable or C^{14} -tagged alpha-methoxyphenylacetic acid containing 1-5 microcuries of radioactivity/mg. for standard treatment
- Distilled water

Suggested plant material—Young snap bean plants (*Phaseolus vulgaris* L.) with primary leaves approximately 4-6 cm. wide and with their trifoliolate leaves still folded in the terminal bud. Other kinds of young plants can also be used with this method.

Preparation and selection of plant material—Plant several bean seeds in composted soil in pots so one plant of uniform size can be selected in each pot. Sever the stems of the undesirable plants at the soil surface and discard the above-ground portions. Submerge the pots in water to the depth of the soil surface for a short period and then carefully wash the soil from the roots of the selected plants in running water. Keep the roots in water until the plants are needed.

Procedure—Construct the apparatus as follows (fig. 25): Fit a 2-hole rubber stopper in the lower end of the 32-mm. diameter glass tubing (cylinder). Connect one of the outlets in the stopper, using the soft glass and plastic tubing (4 mm. inside diameter), with a manifold made by connecting a series of needle valves with



BN-28925

FIGURE 25.—Apparatus for studying exudation of regulating substances applied to leaves of intact plants.

plastic tubing. Make one end of the glass tubing extend 1 cm. above the stopper into the cylinder. Use the other opening in the stopper at the lower end of the cylinder as a drainage outlet by inserting a piece of glass tubing with a short length of rubber tubing attached. Close this outlet with a pinch clamp. Cover the cylinder with aluminum foil leaving an uncovered portion to serve as a window. Support each pair of cylinders with a single ring stand.

Construct a metal plant support by marking the surface of a metal sheet (a tin can is satisfactory) using the pattern shown in fig. 26. Bend the metal into the form illustrated in fig. 27, *c*. Spray these plant supports with aluminum paint to prevent rusting and mount them on cylinders (fig. 27, *d*). Cut pieces of the rubber tubing (3 mm. inside diameter) approximately 5 mm. long (fig. 27, *a*) and pieces of the plastic tubing (5 mm. inside diameter) approximately 20 mm. long (fig. 27, *b*). Cut along the side of each piece of tubing so that it can be opened and fitted around the stem of the plant.

Connect the manifold to the aquarium pump leaving at the end of the manifold an outlet that can be adjusted by a pinch clamp, and thus divert the appropriate amount of air through the glass cylinder. Drill fine holes in a short piece of the plastic tubing to serve as a "bubbler." Plug one end with a short piece of glass rod and attach the other end to the 1-cm. length of glass tubing that extends from the stopper into the cylinder. The air from the pump will be forced through the holes and form small bubbles. Start the aquarium pump and adjust the manifold outlet needle valve and the other needle valves, so air is passing through each cylinder. Introduce 100 ml. of tapwater or nutrient solution into each cylinder. Adjust the rate of airflow so the number of bubbles passing gently through each cylinder is about equal. Carefully place the roots of 1 plant in each cylinder and support the stem of the plant at the proper height with the plastic and rubber collars and metal supports (fig. 25 and 27).

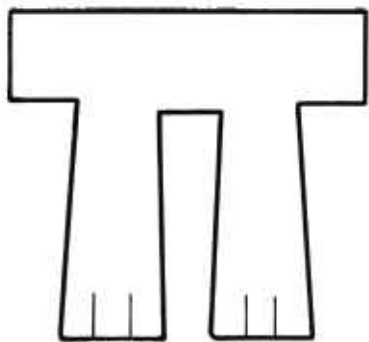


FIGURE 26.—Pattern to be used in cutting metal support. (See also fig. 27, c).

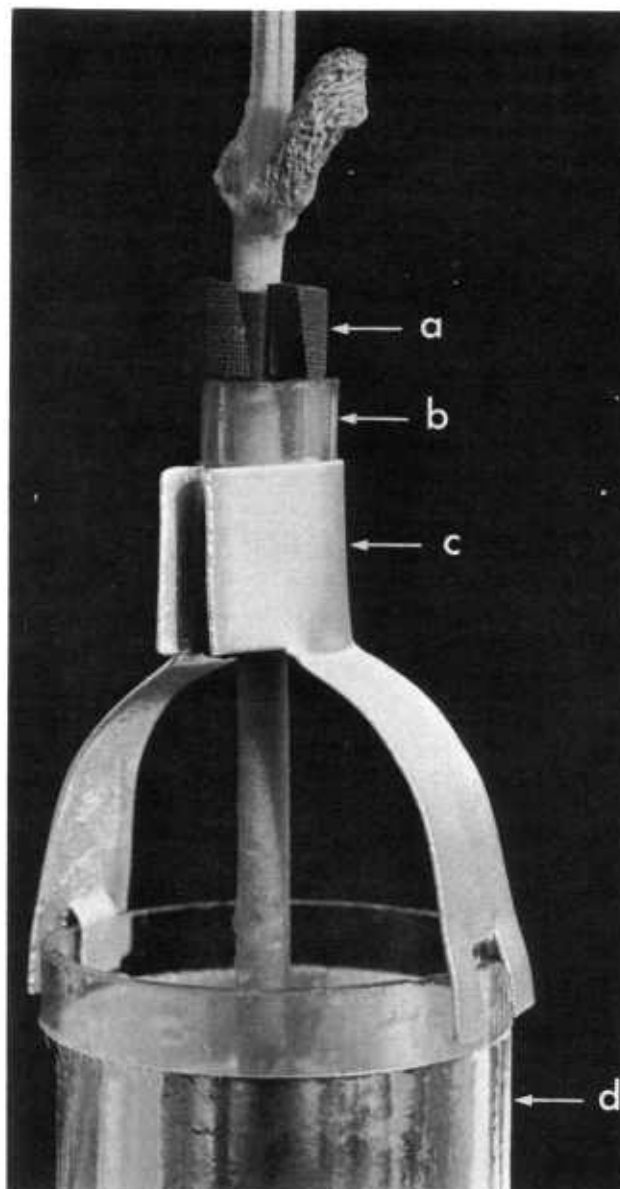
Apply 1–100 micrograms of the chemical being tested to one or both primary leaves as illustrated in fig. 7, p. 12.

Method of taking results—Place a 100-ml. beaker under each cylinder and collect separately the liquid surrounding each root at 12-hr. intervals for several days. Replace the tap-water or nutrient solution in each cylinder. Evaporate the liquid collected and determine the amount of radioactivity in each sample with a Geiger counter. If a nonradioactive compound is used, bioassay the samples using the “Bean second internode method” described on p. 26, the “Wheat coleoptile straight growth method” described on p. 36, or other suitable bioassay method. Estimate the amount of compound exuded by comparing the amount of radioactivity or the biological response to standard curves made with known amounts of the compound involved.

Suggested standard for comparison—10 micrograms of carboxyl-tagged C^{14} or stable alpha-methoxyphenylacetic acid applied to the upper surface of each primary leaf.

Reference

Mitchell, J. W., Linder, P. J., and Robinson, Melba B. Mechanism of Root Exudation of α -Methoxyphenylacetic Acid in the Bean Plant. *Bot. Gaz.* 123: 134–137. 1961.



BN-28924

FIGURE 27.—Plant support: *a*, Rubber tubing, 3 mm. inside diameter; *b*, plastic tubing, 5 mm. inside diameter; *c*, metal support; *d*, glass cylinder covered with aluminum foil.

FLOWERING

BIENNIAL OR PERENNIAL PLANT METHOD

Although research on hastening the time of flowering of biennial and woody plants with regulators is an important field, the methods

used simply involve the application of a regulator in solution to soil surrounding the roots or to the aboveground parts as a paste or spray. The time required for the treated plants to initiate flowers compared with that required for

similar untreated plants is a measure of the effectiveness of the compound. A detailed description of these methods is, therefore, unnecessary here.

References

- Batjer, L. P., Williams, M. W., and Martin, G. C. Effects of *n*-Dimethyl Amino Succinamic Acid (B-Nine) on Vegetative and Fruit Characteristics of Apples, Pears, and Sweet Cherries. *Amer. Soc. Hort. Sci. Proc.* 85: 11-16. 1964.
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- Marth, P. C. Effect of Growth Retardants on Flowering, Fruiting, and Vegetative Growth of Holly (*Ilex*). *Amer. Soc. Hort. Sci. Proc.* 83: 777-781. 1963.
- Stuart, N. W. Stimulation of Flowering in Azaleas and Camellias. XVI Internatl. Hort. Cong. Proc., Brussels, Belgium. 5: 58-64. 1962.

FORM OR GROWTH MODIFICATION

BEAN LEAF METHOD

Based on research by J. W. Brown and R. L. Weintraub.

Description of method—The growth-regulating chemical in a small droplet applied to the terminal bud of bean plants suppresses the vegetative growth of the young leaves, which serves as an index of growth-suppressing activity.

With slight modification, this test can be used to study the effect of regulating chemicals on the formation of abscission layers near the terminal buds of succulent plants.

Apparatus, Chemicals, and Other Materials

Composted soil
Clay pots or other suitable containers in which to grow plants, 10 cm.
Greenhouse or, for more precise experiments, an artificially lighted room (about 700 foot-candles) equipped with temperature and humidity controls
Analytical balance
Pipette, 5 lambda
Photographic paper, printer, and developer
Photometer
Planimeter
200 mg. of each compound to be evaluated
200 mg. of 2,4-dichlorophenoxyacetic acid for standard treatment
95% ethanol
Tween 20 or a similar wetting agent
Sulfuric acid

Suggested plant material—Young bean plants (*Phaseolus vulgaris* L.)—Black Valentine, Red Kidney, Bountiful varieties.

Preparation and selection of plant material—

Plant 3 or 4 bean seeds in soil in each of the containers and grow the plants under greenhouse conditions. If the plants are grown under controlled environmental conditions, employ a day length of approximately 14-16 hr., a minimum of 700 foot-candles of light, a day temperature of about 24° C., a night temperature of about 22° C., and, if possible, a relative humidity of 50-70%. Select test plants for uniformity (1 plant per pot) after 6-8 days when the second internode is 3-7 mm. long. Cut off and discard all other plants.

Procedure—Dissolve a weighed amount of chemical to be tested in a measured volume of solvent mixture composed of 95% ethanol and 1% Tween 20. With the lambda pipette, apply a 5-lambda droplet to the terminal bud of each of 10-20 test plants. Repeat the procedure, using additional plants for each desired dosage level. Apply equal amounts of the solvent mixture to additional plants for comparison, and leave others untreated for controls. Replace all plants in the greenhouse or under the uniform, controlled environmental conditions described until the control plants have developed a partially expanded trifoliolate leaf (5-13 days).

Method of taking results—At the end of this growth period, remove the first trifoliolate leaf from each plant, and determine its fresh weight and area. Leaf area can be obtained directly using an area photometer or indirectly using a planimeter and tracing an outline of a leaf on paper. Also, leaf area can be obtained by tracing the outline of the leaf on paper and cutting out the image. Weigh a piece of the paper that is

exactly 10 cm. square. Determine the weight of the leaf image and calculate its area from the known weight of 1 square cm. of paper. A contact shadowgraph of the leaf made on photographic paper can be substituted for the tracing as used above. Calculate the effect of the chemical on leaf area as percentage increase or decrease in comparison with controls. Record any other differences between leaves on treated and control plants.

Suggested standard for comparison—Use standard dose of 0.015 μ g. of 2,4-dichlorophenoxyacetic acid per 5-lambda droplet per plant. This induces approximately 48% suppression in leaf area.

Reference

Brown, J. W., and Weintraub, R. L. A Leaf-Repression Method for Evaluation of Formative Activity of Plant Growth-Regulating Chemicals. Bot. Gaz. 111: 448-456. 1950.

LANOLIN PASTE METHOD

Based on research by P. C. Marth, W. H. Preston, Jr., and J. W. Mitchell.

Description of method—Compounds are applied in a paste carrier to stems of young plants, and the effect on their subsequent growth and development is observed.

Apparatus, Chemicals, and Other Materials

Clay pots, 10 cm.

Composted soil

Greenhouse space with temperature controlled at 24°–29° C.

Shell vials, about 10-ml. capacity

Wooden applicators that can be used once and discarded (obtainable without cotton from drug- or hospital-supply store)

Analytical balance

100–200 mg. of each chemical to be evaluated

For standard treatment:

50 mg. of 2,4-dichlorophenoxyacetic acid;
or 50 mg. of (4-hydroxy-5-isopropyl-2-methylphenyl) trimethyl ammonium chloride, 1-piperidine-carboxylate (Amo-1618); or 50 mg. of indole-3-acetic acid

Tween 20

Lanolin

Suggested plant material—Young herbaceous plants—bean (*Phaseolus vulgaris* L.), cucumber (*Cucumis sativus* L.), sunflower (*Helianthus annuus* L.).

Preparation and selection of plant material

Plant 3 or 4 seeds of one of the species per pot in the composted soil. Germinate the seeds at about 29° C. and, as soon as the plants appear above the soil, reduce the temperature 2°–5° C. After the plants are erect and about 5–8 cm. tall, select one in each pot that is of average size. Pinch the remaining plants off at the soil level and discard them.

Procedure—Arrange each species of plant in a group with 3–5 plants per row. Prepare lanolin paste as described on p. 8. With an applicator stick, apply a portion of the paste about the size of a wheat seed in the form of a band 3–6 mm. wide around the first internode midway between the first and second nodes of each bean plant in one row. Use the same procedure if cucumber and sunflower plants are employed except place the band on the hypocotyls of these plants just below the cotyledons. Allow the plants to grow for 7–12 days, along with several rows of untreated plants. Additional plants should be treated in a similar manner with the lanolin-Tween 20 paste alone to study the effect of the carriers.

As a modification, using additional plants, apply an equal amount of the paste containing the chemical to be tested to an area about 4 mm. wide and 10 mm. long on the upper surface of one primary leaf of each bean plant and on the upper surface of the first true leaf of the cucumber and sunflower plants. For the most effective treatment, cover an area starting at the junction of the petiole and leaf blade and extending toward the tip of the blade. Cover the midrib and an area extending about 2 mm. on each side of it with the paste.

Method of taking results—For bean and sunflower plants, measure the terminal growth that occurs during the week following treatment by placing the end of a rule at the node immediately above the treated section of the stem and determining the distance from this point to the tip of the terminal bud. Measure the untreated plants in a similar manner. For cucumber plants, place the end of the rule at the cotyledonary node and measure the distance from this point to the tip of the first true leaf, since during the test period the measurable growth occurs almost entirely in this leaf. Measure corresponding control plants in the manner described.

Average the measurements in each test and compute the percentage of inhibition or stimulation of terminal growth of bean and sunflower and leaf growth of cucumber by the following formula:

$$\frac{\text{Untreated control}^3 - \text{minus treated}}{\text{Untreated control}} \times 100 = \begin{array}{l} \% \text{ inhibition} \\ \text{(plus value) or} \\ \text{stimulation} \\ \text{(minus value)} \end{array}$$

During the test, note any differences in the shape of leaves, stems, or other parts that develop, the development of galls indicating cell enlargement or cell proliferation, initiation of roots, changes in color, and other modifications of growth that result from presence of the chemical in the plant. Note the position of the affected parts in relation to the treated area as an indication of transportability of the compound.

Suggested standard for comparison—On the basis of knowledge gained from the literature or previous experience, select several compounds known to be relatively effective in inducing specific responses, such as 2,4-dichlorophenoxyacetic acid for gall formation, translocation, and inhibition of terminal growth; Amo-1618 for suppression of stem growth; and indole-3-acetic acid for gall formation and induction of root primordia on stems. Apply these to the selected test plants as examples of compounds that are relatively effective in inducing these various types of growth modifications.

Reference

Marth, P. C., Preston, W. H., Jr., and Mitchell, J. W. Growth-Controlling Effects of Some Quarternary Ammonium Compounds on Various Species of Plants. Bot. Gaz. 115: 200-204. 1953.

³ Control plants treated as described with the lanolin-Tween 20 paste alone should also be included if the effect of such substances on the test plants is not known.

UNDERGROUND GROWTH METHOD

Based on research by T. J. Muzik and H. J. Cruzado; T. J. Muzik and J. W. Whitworth.

Description of method—Plants are grown in soil in boxes constructed with glass sides in order to observe the effects of chemicals on growth and development of the underground parts without disturbing the plants.

Apparatus, Chemicals, and Other Materials

Wooden or metal boxes, 23 cm. wide at the top \times 96 cm. long \times 43 cm. deep with glass sides slanting inward at an angle of about 60° and with removable wooden, tar paper, metal, or cloth shutters to exclude light

Composted soil

Greenhouse or other space suitable for growing test plants

Small hand sprayer

1-5 g. of each compound to be evaluated

For standard treatment:

1-2 g. of 2,4-dichlorophenoxyacetic acid, maleic hydrazide, or 3-indolebutyric acid, depending upon type of response to be studied

Tween 20 or other suitable solvents and wetting agents for preparing and applying sprays

Lanolin

Analytical balance

Suggested plant material—Plants or plant parts whose underground growth is to be observed, such as seeds, stem pieces, root pieces, bulbs, corms, tubers, and rhizomes.

Preparation and selection of plant material—Select uniform plant parts such as seeds, stem pieces, chains of nutgrass nutlets (*Cyperus rotundus*), rhizomes of bindweed (*Convolvulus arvensis* L.) or of Canada thistle (*Cirsium arvense* Scop.), and plant them in composted soil against the sloping glass sides of the boxes.

Procedure—Apply the chemical to be tested to the plant parts before planting, after emergence, or to the soil surface before emergence. Use a foliage spray application (1,000 p.p.m. suggested), a dust or spray application (range of 0.1-16 lb./acre suggested), or as a paste (0.1-1% in lanolin) applied to the leaves or stems covering areas of 1-4 sq. cm. Leave some entire boxes of plants untreated as controls. Maintain the plants at optimum moisture, light, and temperature conditions for growth of the test species employed.

Method of taking results—Observe the underground parts of treated and untreated plants periodically by removing the shutters. Periodically, record data on root initiation and elongation, death of roots, rate of tuber formation, bud or stem growth, discoloration, and other responses. Comparison of these results with those from the control plants indicates the effectiveness of the compounds for inducing the type of response involved.

Suggested standard for comparison—For standard treatment, compound used will depend upon type of plant response involved. For example, growth suppression, 2,4-dichlorophenoxyacetic acid, maleic hydrazide; root initiation on stem or root pieces, 3-indolebutyric acid.

Variation used by Muzik and Whitworth—Boxes of somewhat different shape have also proved useful. Make glass-faced boxes using marine plywood approximately $\frac{3}{8}$ in. thick. A convenient size has inside dimensions of 5 cm. deep \times 20 cm. wide \times about 1 m. high. Leave one side of the box open and equip this side with grooves so that a glass plate can be fitted into the grooves. Make a close-fitting, removable shutter to cover the glass plate and fasten this in place with clips so that the roots can be kept in darkness.

Fill the box with vermiculite, sand, or soil (preferably vermiculite), and moisten the medium. Place seedlings or rooted cuttings on the surface of the medium with the part ordinarily above the soil level extending out of the open end of the box. Spread the roots out to permit ready observation and place the glass over them. Fasten the wooden shutter over the glass and

place the box at an angle of 30° – 45° with the glass side down.

Using a wax pencil, mark the position of the roots on the glass at frequent intervals to indicate their growth. Use these marks to make final growth measurements. If it is necessary to remove the glass plate during the experiment, wait several hours after watering the plants before doing this because the wet vermiculite adheres to the glass.

This simple technique permits the detailed study of root growth and development with a variety of plants under a wide range of experimental conditions.

References

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FRUIT GROWTH

OVARY CULTURE METHOD

Based on research by J. P. Nitsch.

Description of method—Excised ovaries of various plants are grown *in vitro* on synthetic nutrient medium. Regulating or other chemicals are added, and their effects on growth of the fruits are determined.

Apparatus, Chemicals, and Other Materials

Scalpel
Microbiological transfer room
Jars
Petri dishes
Filter paper
Test tubes
Forceps
Nonabsorbent cotton for test tube plugs
Room exposed to indirect sunlight with temperature of about 23° C. and night temperature of about 17° C.
Analytical balance
Calcium hypochlorite, 5%, c.p. in water
Salts for nutrient solution:
1 g. of $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$
1 g. of KNO_3
1 g. of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
1 g. of KH_2PO_4
Chemicals for trace-element solution:
5 ml. of H_2SO_4 , sp. gr. 1.83
5 g. of $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$

1 g. of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$
1 g. of H_3BO_3
1 g. of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$
1 g. of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$
1 g. ferric citrate
5 g. of NaOH
5–10 ml. of HCl
100 g. of sucrose
500 ml. of 70% ethanol
Paraffin
Water redistilled in Pyrex
Agar
Detergent, 10 ml./liter of water

Suggested plant material—Commercial varieties of crop plants—tomato (*Lycopersicon esculentum* Mill.), bean (*Phaseolus vulgaris* L.), tobacco (*Nicotiana tabacum* L.), strawberry (*Fragaria* spp.), cucumber (*Cucumis sativus* L.), common varieties of other crop plants in flower.

Preparation and selection of plant material—Use healthy greenhouse plants. At flowering, hand-pollinate strawberry and cucumber flowers to insure pollination. Shake tomato, bean, and tobacco plants occasionally to insure pollination.

Cut the flowers from the plants 2 days after pollination and dip the cut ends into melted paraffin to seal them. Place these flowers into clean jars, sterilized by washing with 70% ethanol. Pour a detergent solution over the flow-

ers and then drain the jars. Add a decanted or filtered 5% calcium hypochlorite solution. From then on conduct all operations in a microbiological transfer room, using sterile utensils and materials.

After about 10 minutes, pour the calcium hypochlorite solution off and wash twice with sterile water. Transfer the flowers to sterile petri dishes containing a double thickness of filter paper to absorb the excess water.

Procedure—Prepare the following solutions:

Mineral salt solution

Salt	Amount per liter of final solution
Ca(NO ₃) ₂ • 4 H ₂ O	500 mg.
KNO ₃	125 mg.
MgSO ₄ • 7 H ₂ O	125 mg.
KH ₂ PO ₄	125 mg.

Add sufficient water redistilled in Pyrex to make 1 liter of final solution.

Trace-element solution

Component	Amount per liter of final solution
H ₂ SO ₄ , sp. gr. 1.83	0.5 ml.
MnSO ₄ • 4 H ₂ O	3,000 mg.
ZnSO ₄ • 7 H ₂ O	500 mg.
H ₃ BO ₃	500 mg.
CuSO ₄ • 5 H ₂ O	25 mg.
Na ₂ MoO ₄ • 2 H ₂ O	25 mg.

Add sufficient water redistilled in Pyrex to make 1 liter of final solution.

To 1 liter of the mineral salt solution add 10 mg. of ferric citrate, 1 ml. of the trace-element solution, and 50 g. of sucrose. Adjust the pH to 5.5 with NaOH or HCl.

Divide the medium into separate aliquots and add required amounts of the test chemicals. Three treatments and a control, each having 10 replicates, can be run by dividing 1 liter of nutrient medium into four 250-ml. portions and supplying each ovary with 25 ml. of nutrient medium in separate tubes. To support the ovaries, use filter-paper disks having a diameter about twice that of the tubes. Make a hole in the center of each disk slightly larger than the diameter of the pedicel. Form a paper cup with each disk by pressing it against the edge of a tube. Push one cup inside each tube, edges downward, so it stays at least 5 cm. above the level of the liquid. Plug the tubes with nonabsorbent cotton. Autoclave the tubes 20 minutes at 15 lb. pressure. When planting the sterile ovaries, push the paper down with the forceps until the pedicel reaches the liquid.

A variant of this technique uses no filter paper but solidifies the media with 0.8% agar.

Suggested concentrations of the chemical to be tested are 0.001–100 mg. per liter in serial dilutions.

Due to possible breakdown at high temperatures it is sometimes advisable to avoid autoclaving solutions that contain organic compounds. Addition of test chemicals aseptically to the nutrient medium can be done as follows using agar media: Autoclave the four 250-ml. portions of nutrient medium mentioned previously and the plugged, but empty tubes. Sterilize a metal spoon by dipping it into alcohol, and allowing it to dry. Weigh the required amount of test chemical, place it in the spoon, and add sufficient ethyl alcohol to barely cover the compound. Allow the alcohol to evaporate; then immerse the bowl of the spoon containing the chemical in one of the sterile portions of nutrient medium before the agar has solidified and stir until the compound is uniformly dispersed. Remove the cotton plugs from the tubes and pour approximately 25 ml. of the mixture directly into each tube. Replace the cotton plugs.

Cut off the stamens, petals, two-thirds of the sepals, and most of the pedicel of the sterilized flowers. Cut the pedicel off above the abscission zone (at least 1 mm. below base of flower) to eliminate the abscission zone and prevent the abscission phenomenon from interfering with the growth of the ovary. Care must be taken with tomatoes to avoid any film of liquid remaining between the sepals and the ovary, since this seems to cause the ovary to turn brown and die. Support the sterile, trimmed flower on the disk of filter paper provided with a central hole so that only the pedicel extends into the solution.

Place the tubes containing the ovaries on shelves where they are exposed to indirect sunlight in a room with a day temperature of about 23° C. and a night temperature of about 17° C.

Method of taking results—At intervals measure length and diameter with a small translucent metric rule through the walls of the test tubes. At conclusion of experiment, remove each ovary and accurately measure it. Prepare a table of length and diameter dimensions with corresponding volumes for each specific variety of plant. Use these data for comparing growth rates of treated and untreated fruits.

Suggested standard for comparison—No standard treatment suggested.

References

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FRUIT SET**AEROSOL METHOD**

Based on research by L. D. Goodhue; C. L. Hamner, H. A. Schomer, and P. C. Marth; P. C. Marth and E. M. Meader.

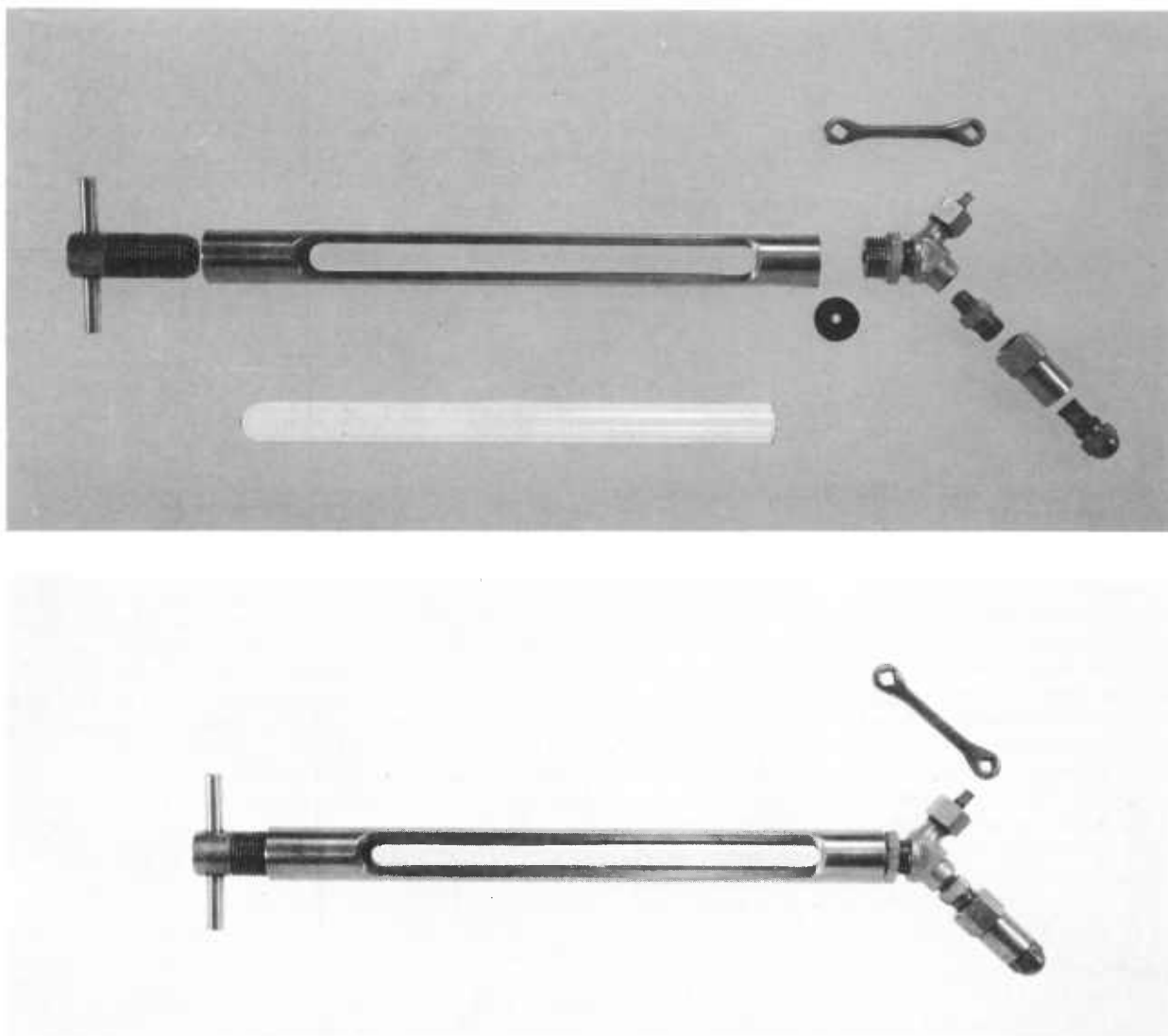
Description of method—Regulating or other chemicals are applied to plants or plant parts in the form of mists produced by use of liquefied gases.

Apparatus, Chemicals, and Other Materials

Test-tube-type apparatus (fig. 28) for small-scale experiments or screening tests

Steel containers 0.23–0.45 kg. capacity, developed for applying insecticidal liquefied gas aerosol mixtures and fitted with 1.27-cm. refrigerant valves

Flexible rubber pressure tubing for transferring liquefied gases



FN-296, A and B

FIGURE 28.—Views of unassembled (*upper*) and assembled (*lower*) glass-walled aerosol bomb of the test-tube type used experimentally to prepare aerosol mixtures of regulating chemicals.

Brass fittings for connecting valves and tubing

Analytical balance

Suction pump or air vacuum line

50-mm. metal funnel soldered into 0.6-cm. threaded brass valve fitting

Spring scales or balance, 1,000-g. capacity

Spray nozzles (standard oil burner nozzles or capillary metal tube soldered into 0.6-cm. thread fitting)

25 mg.—1 g. of each compound to be tested

5.0 g. beta-naphthoxyacetic acid for standard treatment

Dimethyl ether

100–200 ml. of solvents such as cyclohexanone, acetone, or carbitol that are miscible with liquefied gases and will dissolve chemicals to be tested

2.3–11.3 kg. supply tank of methyl chloride, boiling point -11.9°C. ; or dichlorodifluoromethane (Freon), boiling point -6°C. ; or dimethyl ether, boiling point -12.4°C.

Suggested plant material—Tomato plants (*Lycopersicon esculentum* Mill.) that have developed 2 open flowers in the first cluster.

Preparation and selection of plant material—Tomato plants (*Lycopersicon esculentum* Mill.) Marglobe, Globe, Rutgers, or other varieties that can readily be grown in a greenhouse.

Procedure—Preparing and loading aerosol mixtures—

A. For test-tube-type apparatus shown in figure 28: Prepare 25 g. of aerosol mixture containing a 1% concentration of beta-naphthoxyacetic acid as follows: Weigh out 2.5 g. of this acid and place in bottom of the test tube; dissolve this in 2.5 ml. of cyclohexanone (or acetone); place the test tube in holder and seal tightly with basal plug. Connect the valve with a suction pump or vacuum line by means of the pressure tubing and evacuate as much air as possible from the test tube. Close the test tube valve tightly, thus maintaining a partial vacuum. Connect the valve opening of the test tube to the supply tank of methyl chloride or dimethyl ether with pressure tubing. The supply tank must be supported in an inverted position to obtain the liquefied gas. Warm the supply tank gently, using hot water or a small hot plate. Open the valve on the test tube; then slowly open the valve on the supply tank, and allow the liquefied gas to flow out and completely fill the pressure tubing with only a drop or 2 overflowing into the test tube. Close the

test tube valve and weigh the test tube with the pressure tubing still attached to the test tube and the supply tank. Open the valve of the test tube and allow 22–25 g. of the liquefied gas to flow into the test tube. Close the valve of the test tube and the reserve supply tank; then disconnect the pressure tubing from the test tube, and attach the spray nozzle in place of the tubing. Mark the level of the liquid on the test tube for reloading in the future; invert the test tube, and apply the aerosol spray to clusters of tomato flowers. Repeat procedure for each compound to be evaluated. Count the number of flowers and the number of fruit that set (remain attached to the plant) per cluster within 14–20 days after treatment, or before the plants become excessively potbound or the above-soil parts become crowded. Determine the percentage of fruit set by the treated and the untreated clusters.

B. For large (0.23–0.45 kg. or larger) aerosol cylinders: Evacuate the air from the cylinder with a suction pump; close valve; attach funnel to valve and fill with solvent plant-regulator solution prepared as in “A” above; open valve and allow partial vacuum to suck contents of the funnel into cylinder (retain as much partial vacuum as possible within the cylinder), close the valve, and load with liquefied gas, as in “A” above. Conduct test with tomato plants as described. Do not use Freon as most acids are not soluble in it. Freon can be used for dispersing esters and other oil-soluble compounds since they are soluble in it.

Precautions: Methyl chloride gas is toxic to animals and humans, and should not be inhaled. As a safety precaution, prepare mixtures under a ventilated hood, or use a gas mask.

Dimethyl ether forms an explosive, inflammatory mixture with air at about 18% concentration. Load tubes only in well-ventilated rooms and in the absence of open flames.

Freon is noninflammable and relatively non-toxic to plants and animals.

Suggested standard for comparison—Dimethyl ether aerosol mixture containing 1% beta-naphthoxyacetic acid and 10% cyclohexanone solvent for tomato fruit-set test.

References

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Marth, P. C., and Meader, E. M. Influence of Growth-Regulating Chemicals on Blackberry Fruit Development. Amer. Soc. Hort. Sci. Proc. 45: 293-299. 1944.

GRAPE METHOD

Based on research by R. J. Weaver and W. O. Williams; R. J. Weaver; R. J. Weaver and S. B. McCune.

Description of method—Various compounds can be evaluated as growth regulators by dipping individual clusters of grape flowers or the immature fruits in solutions of the compound and recording improvement in fruit set and size.

This test is also applicable for the study of fruit set of many kinds of berries.

Apparatus, Chemicals, and Other Materials

Containers of suitable size, such as discarded but clean tin cans, approximately 1-liter, to hold solutions so the flower clusters can be dipped

Analytical balance

Small labels with string or wire attached

25-100 mg. of each compound to be evaluated

About 0.5 g. of para-chlorophenoxyacetic acid for standard treatment or 0.5 g. of gibberellic acid

Wetting agent such as Dreft, Tween 20, Santomerse

Solvent such as 95% ethanol

Suggested plant material—Seedless grape varieties, such as Black Corinth (*Vitis vinifera* L.), or seeded grapes that ordinarily set few berries, such as Muscat of Alexandria, Pinot Chardonnay.

Preparation and selection of plant material—Select clusters of uniform size having about the same number of flowers that have attained the same stage of development. Tag the selected clusters and prepare to apply treatments when the flowers are in full bloom or within 10 days thereafter.

Procedure—Prepare aqueous solutions of each compound at concentrations of 5, 15, and 50 p.p.m. by first dissolving the required amount of the regulator in 0.5-1 ml. of alcohol and then adding the alcohol solution to the required amount of water. Add sufficient wetting agent to make a final concentration of 0.05-0.1% of the detergent.

Dip the clusters of flowers when they are in full bloom or within 10 days thereafter. Do not

bruise or damage these clusters. Use 10 clusters per treatment and leave comparable labeled clusters untreated for controls. Additional clusters should be dipped in a solution containing the solvents and the wetting agent to determine the effect of these constituents.

Method of taking results—Determine the percentage of fruit set by first counting the number of flowers immediately after dipping them. After the fruits are relatively mature, count the number of berries present. Calculate the percentage of fruits that developed from the flowers. Compare this figure with that of the controls.

Suggested standard for comparison—5, 15, and 50 p.p.m. of para-chlorophenoxyacetic acid, or 10, 50, and 100 p.p.m. of gibberellic acid.

References

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Weaver, R. J., and McCune, S. B. Response of Certain Varieties of *Vitis vinifera* to Gibberellin. Hilgardia 28(13): 297-350. 1959.

TOMATO METHOD

Based on research by D. D. Hemphill; F. S. Howlett; L. C. Luckwill; A. E. Murneek, S. H. Wittwer, and D. D. Hemphill; R. H. Roberts and B. E. Struckmeyer.

Description of method—Compounds are applied in liquid or paste carriers to flower clusters of greenhouse plants, and the effectiveness of the substances in preventing flower or fruit drop is evaluated.

This method is also adapted to studies of the effect of regulating compounds on seed development and the morphological development of fruits. It is also useful in studying the effect of regulators on the number of flowers produced.

Apparatus, Chemicals, and Other Materials

Clay pots or discarded tin cans, 0.95 liter with perforated bottoms

Composted soil

Space in greenhouse maintained at about 21°–27° C. by day and at 15°–21° C. by night

Small vials, 20–25 ml.

Clean containers, 100–200 ml. capacity

Toothpicks or wooden medicinal applicators or a nasal spray atomizer, 15–30 ml.

Analytical balance

25–100 mg. of each compound to be evaluated

About 0.5 g. para-chlorophenoxyacetic acid or beta-naphthoxyacetic acid for standard treatment

Lanolin

Solvents such as ethanol, methanol

Wetting agents such as Tween 20, Carbowax 1500

Suggested plant material—Tomato plants (*Lycopersicon esculentum* Mill.), Marglobe, Globe, Rutgers, or other varieties that can be readily grown in a greenhouse.

Preparation and selection of plant material—

A. For tests of limited duration (14–20 days): Grow the plants in 10- to 15-cm. pots set side by side in rows 10 cm. apart; select plants of uniform size which have developed 2 open flowers in the first cluster.

B. For prolonged tests involving maturation of the fruit (35–45 days): Grow the plants in 25- to 30-cm. pots, or grow them in ground beds. In either case select uniform plants that have developed 2 open flowers in the first cluster. The plants should be at least 30 cm. apart, in rows 60 cm. apart.

Procedure—

A. Paste Method: Prepare a lanolin-Tween 20 paste containing 1% of the chemical. (See p. 8.) Apply a narrow band of the mixture around the stalk (peduncle) of the first flower cluster and about 1–2 cm. from the main stem of the plant. To obtain reliable data, apply one treatment to an individual row of plants extending across the bench or ground bed. Repeat the procedure using additional treatments and corresponding rows. Leave an additional row of plants untreated to serve as controls. When all the treatments have been applied and the control row has been designated, an experimental block has been completed. Repeat the procedure making a second, third, and fourth block, randomizing the relative positions of each treatment within each block.

B. Spray Method: Prepare aqueous mixtures to obtain a concentration range of 10, 20, 40, or

80 p.p.m. of the compound to be tested. (See p. 100.) Use the above experimental plan and spray the mixture on the flower cluster with a nasal atomizer until the liquid drips from each flower. Spray flowers of control plants in a similar way, using water and the co-solvent only.

C. Droplet Method: A variation of the spray method is the application of a droplet onto the individual ovaries of 2 flowers in the cluster using a 1-ml. capacity hypodermic syringe fitted with a No. 17 needle. (Method by L. C. Luckwill.)

Method of taking results—

A. Plants used in tests of limited duration: Count the number of flowers and the number of fruits that set (remain attached to the plant) per cluster within 14–20 days after treatment, or before the plants become excessively potbound or the above-soil parts crowded. Determine the percentage of fruits that set. Cut the green fruits midway and at right angles to their axes, and record the relative amount of gelatinous pulp and the relative numbers of seeds present.

B. Plants used in prolonged tests: Record data as described above and, in addition, record the number of days required for the fruit to develop a pink or red color.

If needed, use the second and third flower clusters on each plant to repeat the earlier treatments. Plants in ground beds may develop 5 or 6 flower clusters that are suitable for treatment. In all instances, apply only one concentration of one chemical to a plant.

Suggested standard for comparison—A paste of 0.1% lanolin-Tween 20 or a spray mixture containing 30 p.p.m. of para-chlorophenoxyacetic acid; a paste of 0.3% beta-naphthoxyacetic acid, or a spray at 100 p.p.m.

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FRUIT SIZE

GRAPE METHOD

Based on research by R. J. Weaver and W. O. Williams; R. J. Weaver; R. J. Weaver and S. B. McCune.

Description of methods—Various compounds may be evaluated as growth regulators by dipping individual clusters of grape flowers at bloom, or after the shatter of impotent berries following bloom, in solutions of the compound, and by recording improvement in fruit size.

This test is useful in studying the effect of plant regulators on the growth of a variety of different kinds of fruits. It is also a useful means of studying the effect of regulating chemicals on the production of parthenocarpic fruit such as tomatoes and blackberries.

Apparatus, Chemicals, and Other Materials

See p. 62.

Suggested plant material—Seedless grape varieties such as Black Corinth, Thompson Seedless, Sultana, Black Monukka (*Vitis vinifera* L.). Seedless varieties are more responsive than are seeded ones for this test.

Preparation and selection of plant material—Select clusters of uniform size having about the same number of flowers. Tag those to be used after the calyptres have fallen. The best time to treat grapes for studies on increasing the

size of the berry is after the shatter of impotent berries following bloom.

Procedure—Prepare aqueous solutions of each compound at concentrations of 5, 15, and 50 p.p.m. using alcohol as a solvent and adding a wetting agent. (See p. 100.) Dip the flower clusters in the solutions soon after the flowers are fully open. Use 10 clusters per treatment and leave comparable, labeled clusters untreated for controls. Additional clusters should be dipped in a solution containing the solvents and wetting agent to determine the effect of these constituents.

Method of taking results—Determine the effect of treatment on berry size by removing the berries from the plants after they are mature and obtaining the fresh weight of 200 fruits selected at random from each treatment. Volume measurements of the fruits may also be determined by the water displacement method. Compare this weight with the results of similar measurements using control fruits. Note any difference in time of maturity or other effects due to treatments.

Suggested standard for comparison—5, 15, and 50 p.p.m. of para-chlorophenoxyacetic acid, or 10, 50, and 100 p.p.m. of gibberellic acid.

References

Weaver, R. J. Response of Certain Varieties of Grapes to Plant Growth-Regulators. *Bot. Gaz.* 113: 75-85. 1951.
See also, "References," p. 62.

FRUIT THINNING

APPLE METHOD

Based on research by L. P. Batjer; C. P. Harley, H. H. Moon, and L. O. Regeimbal.

Description of method—Compounds are evaluated as a means of thinning fruit by applying regulating chemicals to selected limbs of trees 1-3 wk. after full bloom.

By modifying the dosage, the method may be useful in entirely removing undesirable fruits of such trees as the ginkgo, mulberry, and hickory without injury to the trees.

Apparatus, Chemicals, and Other Materials

Analytical balance

For whole-tree experiments, use standard orchard-spray equipment such as is used for applying insecticidal or fungicidal sprays under high pressure (400-800 lb. pressure per sq. in.)

Sprayers having a large tank divided into several 50- or 100-gal. compartments facilitate the work

Hand-pressure sprayer (11-liter) to use if individual limbs are to be sprayed

Hand tally registers, 2 or 3 per person, to record data

10-20 g. of alpha-naphthaleneacetamide or 1-naphthyl N-methyl carbamate for standard treatment

Tween 20 or other wetting agent

Suggested plant material—Apple trees (*Malus sylvestris* Mill., also called *Pyrus malus* L.), any variety in need of thinning.

Preparation and selection of plant material—For whole-tree experiments, select and tag trees of uniform size that have produced about the same number of flowers per tree. Five trees are suggested for each treatment.

If individual limbs are to be used, select on comparable trees outer limbs of about the same size and bearing about the same number of blossoms at the desired stage of development. Apply each treatment to a single limb selected at random from each of 5 or more trees. In this way several treatments can be used on each tree.

Procedure—Prepare aqueous sprays containing several concentrations of each chemical (10, 30, and 60 p.p.m. are suggested). (See p. 100.) Apply sprays 1–3 wk. after full bloom and wet thoroughly all parts of the tree or branch. About 3.8 liters for each year of age is required for spraying entire trees. Leave unsprayed trees or branches as controls, and hand-thin other trees or branches to have trees or branches thinned an optimum amount for comparison.

Method of taking results—Determine the number of fruits thinned chemically after June drop. With the aid of two tally registers, one in each hand, record separately the total number of spurs that blossomed and the number that set fruits. Spurs from which fruits have dropped are easily identified by the pedicel scars. Use a third tally register to record vegetative spurs if desired. Compare the number of spurs that bear fruits with the number from which fruits have fallen. Compare ratios thus obtained with those of the untreated and the hand-thinned controls.

Suggested standard for comparison—50 p.p.m. spray concentration of alpha-naphthaleneacetamide. 3 g./gal. of 1-naphthyl N-methyl carbamate.

References

- Batjer, L. P. Fruit Thinning With Chemicals. U.S. Dept. Agr., Agr. Inf. Bul. 289. 1965.
 Harley, C. P., Moon, H. H., and Regeimbal, L. O. A Modified Method of Obtaining Fruit-Set Records for Apples. Amer. Soc. Hort. Sci. Proc. 63: 146–148. 1954.

PEACH AND PRUNE METHOD

Based on research by P. C. Marth and V. E. Prince.

Description of method—A method of evaluating the fruit-thinning effects of chemicals applied as sprays to limbs or entire trees is described.

By modifying the dosage used, these methods may be useful in entirely removing undesirable fruits of such trees as ginkgo, mulberry, and hickory, without injury to the trees.

Apparatus, Chemicals, and Other Materials

See p. 64, substituting 50–100 g. of isopropyl N-(3-chlorophenyl) carbamate for standard treatment. ("Apple method")

Suggested plant material—Peach trees (*Prunus persica* (L.) Batsch) bearing young fruits (30–45 days after full bloom)—Elberta, Halehaven, Hiley, or other varieties; or plum trees (*Prunus domestica* L.) bearing young fruits (30–45 days after full bloom)—Stanley, Italian, or German varieties.

Preparation and selection of plant material—See "Apple method," p. 64.

Procedure—Prepare aqueous sprays containing several concentrations of each chemical. (See p. 100.) 125, 250, and 500 p.p.m. are suggested. Apply sprays about 30–45 days after full bloom or 10–20 days after shucks (dried flower parts) have fallen. Wet all parts of the tree or branch thoroughly. At the time the fruits are sprayed they should be about 1–2 cm. in diameter. About 3.8 liters for each year of age is required for spraying entire trees. Leave unsprayed trees or branches as controls; also hand-thin other trees or branches to leave an optimum number of fruits for further comparison.

Method of taking results—Within a day or two after applying the sprays, determine the number of fruits that remain attached. After June drop, determine the number of fruits thinned by comparing the number on sprayed, unsprayed, and hand-thinned branches or trees. At the usual harvest date record the number, size, degree of coloration of fruits, and effect of treatment on time of maturation. Compare these data to evaluate effectiveness of the chemical treatment.

Suggested standard for comparison—Spray concentration of 200 p.p.m. of isopropyl N-(3-chlorophenyl) carbamate.

Reference

- Marth, P. C., and Prince, V. E. Effect of 3-Chloro-isopropyl-N-phenyl Carbamate on Abscission of Young Fruits of Peach. Science 117: 497–498. 1953.

GROWTH MODIFICATION

STERILE NUTRIENT METHOD

Based on research by L. G. Nickell.

Description of method—Duckweed is grown under aseptic and rigidly controlled conditions in a nutrient solution. Regulating chemicals (including antibiotics) are added to the nutrient, and their effect on growth of the plant is studied.

Apparatus, Chemicals, and Other Materials

Analytical balance

Petri dishes

Tank in which to maintain a stock supply of the test plant. (*Lemna minor* will grow vigorously through the year in dilute nutrient solutions at a pH of 5–6 or in tap-water over a small amount of sandy soil.)

Table space in a room with constant light intensity of 700 to 1,000 foot-candles for 12 hr. per day and with temperature controlled at 24°–27° C.

0.2 g. of each compound to be evaluated

0.5–1 g. of 2,4-dichlorophenoxyacetic acid in water-soluble salt form for standard treatment

Basic nutrient medium containing the ingredients listed:

KNO ₃	0.002M	Pyridoxine	800 γ /liter
Ca(NO ₃) ₂	0.003M	Nicotinamide	800 γ /liter
KH ₂ PO ₄	0.001M	B	0.1 p.p.m.
MgSO ₄	0.001M	Mn	0.1 p.p.m.
CaCl ₂	0.003M	Zn	0.3 p.p.m.
KCl	0.002M	Cu	0.1 p.p.m.
MgCl ₂	0.001M	Mo	0.1 p.p.m.
Sucrose	2%	Fe	0.5 p.p.m.
Thiamine	100 γ /liter		

Dilute sodium hydroxide and hydrochloric acid solutions

Glass or other type filters with which solutions can be filtered free of contaminating organisms

Suction flasks with adapters for filters

Erlenmeyer flasks, 125 ml.

Tweezers

Cotton

Bacitracin, Penicillin G, or isonicotinic hydrazide for standard treatment

Solution of mercuric chloride (1:2,000 of water) for sterilizing plants

O.T. Clear Aerosol or comparable detergent

Suggested plant material—Duckweed plants (*Lemna minor* L.).

Preparation and selection of plant material—Select a vigorously growing supply of duckweed. Sterilize this material by immersing rosettes (3 or 4 fronds) in the mercuric chloride solution (1:2,000 water). Add one drop of a 1:20 dilution of O.T. Clear Aerosol to each 100 ml. of the mercuric solution. Place several rosettes in the solution and remove them one by one at 5-minute intervals so a series of treated rosettes results which have been exposed from 5–30 minutes to the mercuric chloride mixture. As each rosette is removed, rinse it several times in sterile water and finally place it in a flask containing sterile nutrient medium. Allow all the plants to grow in the flasks for a week to determine which plants are aseptic. Uncontaminated cultures are used for further experimentation.

Procedure—The pH of the basic medium should be about 5.0. Adjust any variation from this with dilute acid or alkali solutions. Prepare 50–100 ml. of an aqueous stock solution of the regulator to be tested so it contains 300 p.p.m. of the chemical. Remove contaminating organisms in this solution by passing it through the sintered glass filter. Place 50 ml. of the sterile nutrient medium aseptically into each of 5 flasks with a 125-ml. capacity. Introduce one sterile duckweed plant into each flask and designate these as controls. Repeat the procedure but, before placing a plant in the nutrient, add a sufficient volume of the sterile stock solution containing the regulating chemical to make a final concentration of 1 p.p.m. Prepare other concentrations of the test chemical in additional aliquots of the nutrient (5, 10, 20, 30 p.p.m. etc.). For each concentration level, use at least 5 replicate flasks. Place one of the sterile, selected, duckweed plants in each flask aseptically. Stopper the flasks with cotton and place them under controlled environmental conditions, 22.5° C. and with light from white or Daylite fluorescent tubes having a constant intensity of at least 700 foot-candles.

Method of taking results—After the required period of growth (1–2 wk.), remove the test plants, blot them free of water, and for each flask record the number of plants and the number of fronds on each. Immediately obtain the fresh weight of all plants from each flask, weighing to the nearest milligram. Compare any increase or decrease in growth of control

and treated plants in terms of number of plants and fronds, and the average weight per plant.

Suggested standard for comparison—Bacitracin, Penicillin G, or isonicotinic hydrazide at a concentration of 20 p.p.m.

References

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WATER CULTURE METHOD

Based on research by H. R. Offord.

Description of method—An aquatic plant, duckweed, is grown in water containing a regulating compound, and the growth-modifying effect of the chemical is observed.

Apparatus, Chemicals, and Other Materials

Analytical balance

Petri dishes

Tank in which to maintain a stock supply of the test plant. Duckweed will grow vigorously through the year in dilute nutrient solutions at a pH of 5-6 or in tapwater over a small amount of sandy soil.

Table space in a room with constant light intensity of 700-1,000 foot-candles for 12 hr. per day and temperature controlled at 24°-27° C.

0.2 g. of each compound to be evaluated

0.5-1 g. of 2,4-dichlorophenoxyacetic acid in water-soluble salt form for standard treatment

Dilute solution of sulfuric acid

Dilute solution of potassium hydroxide

Suggested plant material—Duckweed plants (*Lemna minor* L.).

Preparation and selection of plant material—Select relatively large, uniformly green plants that are growing vigorously.

Procedure—Prepare tapwater solutions (150 ml.) of each compound in a range of concentrations from 0.001-1,000 p.p.m. Place 50 ml. of each concentration in each of 3 petri dishes. Place the same number of duckweed plants in each test solution, using 10 or more for each. Include some dishes that contain plants in tapwater as controls. Record the number of plants, the total number of fronds, and the time of immersion for each culture. Using dilute sulfuric acid or potassium hydroxide, adjust the pH of all solutions to approximately the same level (about 5-6), since excess acid or alkali exerts a toxic action on duckweed. Maintain the temperature of the solutions at 24°-27° C. and the light intensity constant so tests carried out at different times of the year can be compared.

Method of taking results—At regular intervals, record any visible differences in the plants such as change in color, presence or absence of vegetative division, and presence or absence of new fronds. Evaluate the relative effectiveness of each concentration of each compound tested, as follows:

Marked inhibitory effect: Color change becomes steadily more apparent until a uniform discoloration of the fronds is evident. The typical color of fronds in this category is reddish brown to pale yellow or white. There is no change in total area of fronds or in number of plants.

Moderate inhibitory effect: Plants divide and form single or double fronds before discoloration becomes noticeable. Most or all of the plants split into single fronds. Chlorosis occurs slowly, but uniformly, on all fronds, and uniform discoloration results. Typical color of the plants is pale or yellowish green. There is no change in the total area of the fronds.

Slight inhibitory effect: Symptoms appear slowly. Single fronds from parent plants may be unevenly discolored, but fronds of the parent plants remain fairly green. No change is apparent in the total area of the fronds.

No inhibitory effect: Plants are divided into single or double fronds and are dark green. There is an apparent increase in the number of plants and in the total estimated area of the fronds.

Suggested standard for comparison—Determine by trial the minimum concentration of 2,4-dichlorophenoxyacetic acid that induces *marked inhibitory effect*. No standard suggested for stimulation of growth.

Reference

Offord, H. R. Rapid Estimation of the Phytocidal Action of Chemicals. *Science* 103: 474-476. 1946.

FIELD METHOD

Based on research by J. R. Havis; P. C. Marth and R. E. Wester.

Description of method—Test chemicals are sprayed on soil in which seeds have been planted or on leaves and stems of plants growing under field conditions, and the effect of the chemical on growth and productivity of the plants is measured.

Apparatus, Chemicals, and Other Materials

Analytical balance

Knapsack sprayer, to be operated at 30-lb. pressure per sq. in.

5-50 g. of each compound to be evaluated as growth regulator

5 g. of 2,4,5-trichlorophenoxyacetic acid or 2,4-dichlorophenoxyacetic acid for standard treatment

Solvent for preparing concentrated solutions of chemicals to be evaluated (alcohol, acetone, or other organic solvent)

Suggested plant material—Pieces of stem of sugarcane (*Saccharum officinarum* L.) such as are ordinarily used for planting this crop; or seeds of crop plants—corn (*Zea mays* L.), wheat (*Triticum aestivum* L., also called *T. sativum* Lam. and *T. vulgare* Vill.), bean (*Phaseolus vulgaris* L.), soybean (*Glycine max* [L.] Merr.), lima bean (*Phaseolus lunatus* L.), pea (*Pisum sativum* L.), or others.

Preparation and selection of plant material—Plant seeds of crop plants or uniform stem pieces of sugarcane in the usual manner. For

tall-growing crops such as corn or sugarcane, use 4 or more 40-sq.-m. plots for each chemical or concentration tested. Space the plants 46-60 cm. apart in rows 101-106 cm. apart. For short-growing crops such as soybeans and peas, space the plants 15-20 cm. apart in rows 76-91 cm. apart. Use 2 or more replications of rows 1.5-3 m. long for each chemical or concentration tested. It is sometimes advantageous to select uniform plants 60 cm. or more apart. Mark these and use 10 of them for each chemical or concentration tested.

Procedure—For preemergence application, apply the test chemicals within a few days after planting, using suggested rates of 1, 2, 4, and 16 lb./acre.

For postemergence treatment, apply the sprays (0.001-1,000 p.p.m.) at any selected stage of development after emergence. Since the development of this early method, logarithmic sprayers have become available which automatically apply a range of dosage levels.

Method of taking results—Obtain growth and yield data by making linear measurements of at least 10 individual plants in each treatment during the growing period. Also obtain yield of fruits or seeds as they mature. In preemergence experiments, plants near the center of each plot should be measured.

Suggested standard for comparison—Aqueous sprays at concentrations of 0.001-1,000 p.p.m. of 2,4,5-trichlorophenoxyacetic acid or 2,4-dichlorophenoxyacetic acid if applied postemergence; or 0.5, 1, 2, and 7 kg. per 4.047 sq. m. if applied preemergence.

References

- Havis, J. R. Effect of 2,4-D Sprays on the Growth of Sugarcane. *Weeds* 2: 148-154. 1953.
 Marth, P. C., and Wester, R. E. Effect of 2,4,5-Trichlorophenoxy-Acetic Acid on Flowering and Vegetative Growth of Fordhook 242 Bush Lima Beans. *Amer. Soc. Hort. Sci. Proc.* 63: 325-328. 1954.

GROWTH RETARDATION
VEGETATIVE SPROUT METHOD

Based on research by P. C. Marth and E. S. Schultz; P. C. Marth.

Description of method—Pieces of carrots, small onions, and potato tubers are dipped in a solution of the test compound and placed in darkness. Measurement of sprouts indicates the

effectiveness of the compound as a sprout inhibitor.

This test may also be adapted to study the growth and development of buds on any vegetative reproductive organ.

Apparatus, Chemicals, and Other Materials

Cutting spoon, 1 cm., such as one used for making melon balls for salad

Analytical balance

Sphagnum, peat moss, or fine quartz sand
Petri dishes or other shallow containers about 10 cm. in diameter

Space in darkened room with temperature controlled at 18°–24° C.

0.1–0.5 g. of each compound to be evaluated

0.5 g. of methyl ester of alpha-naphthaleneacetic acid; diethanolamine salt of 6-hydroxy-3-(2H)-pyridazinone (MH-30); or 3-chloro-isopropyl-N-phenyl carbamate for standard treatment.

Suggested plant material—Unsprouted vegetable storage organs such as carrots (*Daucus carota* L.), onions (*Allium cepa* L.), and potato tubers (*Solanum tuberosum* L.) that have experienced a rest period.

Preparation and selection of plant material—Carrot: Select carrots of about medium size for uniformity. Sever petioles so about 1 cm. of each petiole remains attached to each root. Remove and discard the lower part of the root saving about 2.5 cm. of the upper part. Place pieces in humid air at 4°–10° C. for 3–4 days or until they become suberized.

Onion: Select small onions about 1 cm. in diameter and of uniform shape. Remove the loose outer scales.

Potato tuber: Select tubers of medium size and, with a cutting spoon, remove pieces of the tubers so that each piece contains an eye (fig. 29). Place the pieces in humid air at 4°–10° C. for several days until they become suberized.



PN-297 A

FIGURE 29.—Use of melon-ball cutting spoon to remove eyes from potato tuber.

Procedure—Select 10 of the prepared carrot pieces. Dip these momentarily in a tapwater mixture containing the desired amount of the chemical being tested (1,000 p.p.m. suggested). Allow pieces to drain, and place equal numbers of them upright on the moist sphagnum, peat moss, or sand contained in dishes about 10 cm. in diameter and without covers (fig. 30). Dip comparable batches of carrot pieces in tapwater and place them on sphagnum or sand in the manner described to serve as controls. Keep the moss or sand moist, and allow the plant material to grow in darkness at room temperature, 18°–24° C.

Apply the chemical to the onion sets and the potato pieces, and culture them in the same manner as for carrot pieces.

Method of taking results—After the controls have developed a measurable amount of new vegetative growth, measure the length of each sprout and the fresh weight of the detached sprouts. Compare these measurements with similar ones for the treated material.

Suggested standard for comparison—1,000 p.p.m. of methyl ester of alpha-naphthaleneacetic acid; 1,000 p.p.m. of diethanolamine salt of 6-hydroxy-3-(2H)-pyridazinone (MH-30); 2,000 p.p.m. of 3-chloro-isopropyl-N-phenyl carbamate.

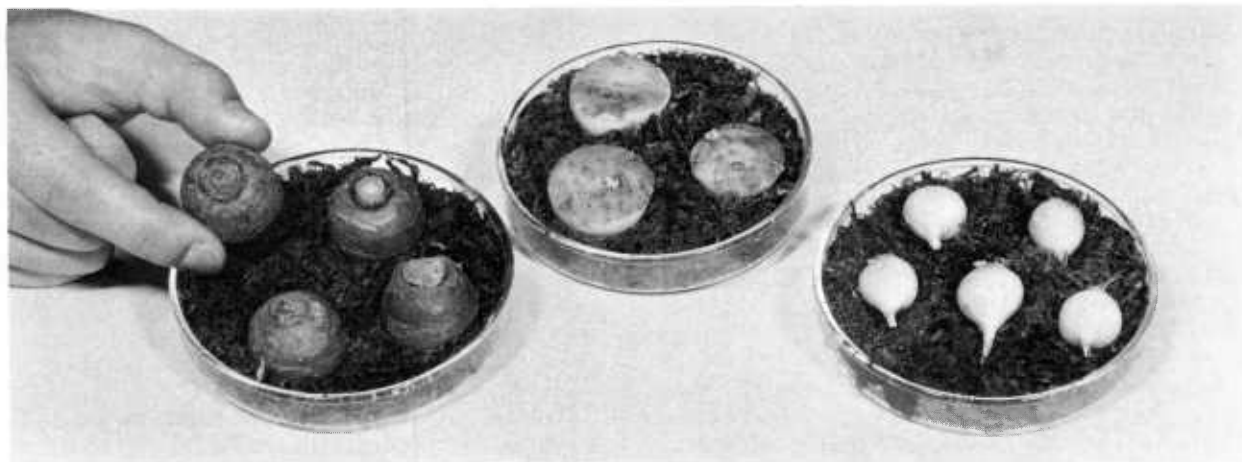
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Marth, P. C., and Schultz, E. S. Effect of Growth Regulators on Sprouting of Stored Table Stock Potatoes and on Waste Piles for Control of Diseases. *Amer. Potato Jour.* 27: 23–32. 1950.
Marth, P. C. Unpublished data, Plant Industry Station, Beltsville, Md.

WHEAT SEEDLING METHOD

Based on research by H. F. Taylor and B. E. A. Knight.

Description of method—The growth-retarding activity of a compound is assessed by comparing the height of wheat seedlings grown in nutrient solution to which the compound has been added with the height of seedlings grown in nutrient solution alone. The seedlings are placed on pieces of gauze made buoyant with polystyrene beads. After the first leaf has emerged from the coleoptile, each gauze “raft” containing the seedlings is floated on the test solution. After 13 days, measurements taken of plants



PN-297 B

FIGURE 30.—Left to right, Carrot roots, potato buds, and onion sets growing on moistened sphagnum moss contained in petri dishes.

grown in the test solution are compared with those of plants grown in solutions without the growth-retarding chemical.

Apparatus, Chemicals, and Other Materials

Filter paper, 9 cm. in diameter
 Petri dishes, 10 cm. in diameter
 Table space in a room illuminated with red light of low intensity and temperature controlled at 24° C.
 Constant humidity boxes with glass covers
 Forceps
 Crystallizing dishes, 94 × 50 mm., unspouted
 Plastic drinking cups, 10 cm. high
 Razor blades
 Circular pieces of coarse cotton gauze, 95 mm. in diameter
 Washed, expanded polystyrene beads, 1–5 mm.
 Growth room with controlled temperature, humidity, and day length
 Analytical balance
 Transparent 50-mm. rule
 Salts for nutrient and micronutrient solutions (See "Procedure")
 100 mg. of each compound to be tested
 1 g. of (2-chloroethyl) trimethylammonium chloride (known as CCC) for standard treatment
 Distilled water

Suggested plant material—Germinated wheat seeds (*Triticum aestivum* L.) Eclipse variety.

Preparation and selection of plant material—Soak wheat seeds in distilled water for 2 hr. and

place them on wet filter paper in petri dishes. Allow the seeds to germinate for 42 hr. in a humidity box (relative humidity 80–85%) at 24° C. and in red light of low intensity. Make a raft upon which the plants will be grown. To do this, make 3 cuts around the plastic cup at the position of the arrows to detach rings *a* and *b* (fig. 31, A). Place the circular piece of gauze (fig. 31, B, *g*) on rings *a* and *b* inverted in the position shown. Press the rings together holding the gauze taut (fig. 31, C). Make the raft buoyant by introducing expanded polystyrene beads beneath the gauze. To do this, proceed as shown diagrammatically in figure 31, D and E. Place the raft (fig. 31, D, *r*) over a small perforated plastic cup (fig. 31, D, *p*) containing dry polystyrene beads. Immerse the raft slowly in water, allowing the cup to be removed and the beads to float up into the raft without trapping large air bubbles. Lift the raft from the water and place it in a crystallizing dish (fig. 31, E, *d*) containing 200 ml. of distilled water (fig. 31, E, *w*). Place 12 uniform 42-hr.-old wheat seedlings around the edge of the gauze with the primary root passing through the gauze to hold the seeds in place. Wrap a black paper band (fig. 31, E, *s*) around the dish to reduce the light intensity to which the roots are exposed and then move the dish to the growth room (23° C., relative humidity 80%, 500–600 foot-candles of light intensity at bench level, day length 15 hr.). Place a filter paper disk over each dish for the first 24 hr. to shade the root tips until they grow through the gauze. When 10–15 mm. of the first leaf has grown through the coleoptile, remove the 2 least uniform plants from each raft.

Procedure—Prepare the following solutions:

Test solution: 100 ml. of solution at a concentration of 10^{-3} M of each compound to be tested.

Nutrient solution: Dissolve in 1 liter of distilled water:

33 g. of KH_2PO_4
133 g. of $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$
17 g. of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
6.1 g. of Fe-EDTA

Micronutrient solution: Dissolve in 1 liter of distilled water:

290 mg. of H_3PO_4
200 mg. of $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$
10 mg. of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$
20 mg. of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$
10 mg. of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$

Dilute the 10^{-3} M test solutions mentioned above with distilled water to give 200 ml. of solution at each of the concentrations to be used, usually 10^{-6} M– 10^{-4} M. Pour each of these solutions into a crystallizing dish and add a raft bearing 10 wheat seedlings.

After 4 days, introduce the nutrients into the culture medium. Replace the solution in each dish with 200 ml. of fresh test solution, which also incorporates 2 ml. of the nutrient solution and 2 ml. of the micronutrient solution. This change is readily achieved either by si-

phoning away the remaining liquid before pouring in the fresh solution, or simply by transferring the raft to a clean dish containing the fresh solution. Conclude the test 9 days later, after the plants have grown in the test solution for a total of 13 days.

Method of taking results—Assess the growth of each of the 10 plants in a dish by measuring the distance between the ligules of the first and second leaves (fig. 31, *F*, x). Compare the “treatment mean” obtained from these 10 measurements with the “control mean” obtained from measurements taken from 10 plants that had received distilled water in place of the test solution. If the treatment mean is subtracted from the control mean and the difference expressed as a percentage of the control mean, a convenient percent retardation value is obtained. Where the base of the second leaf blade has not emerged from the sheath of the first leaf, negative values for x are obtained and figures for the retardation will be greater than 100%.

An alternative method of assessment, which avoids negative values and percentages greater than 100, is as follows: Record the length y (fig. 31, *F*). Obtain treatment means and control means as before and express the difference between these means as a percentage of the control mean.

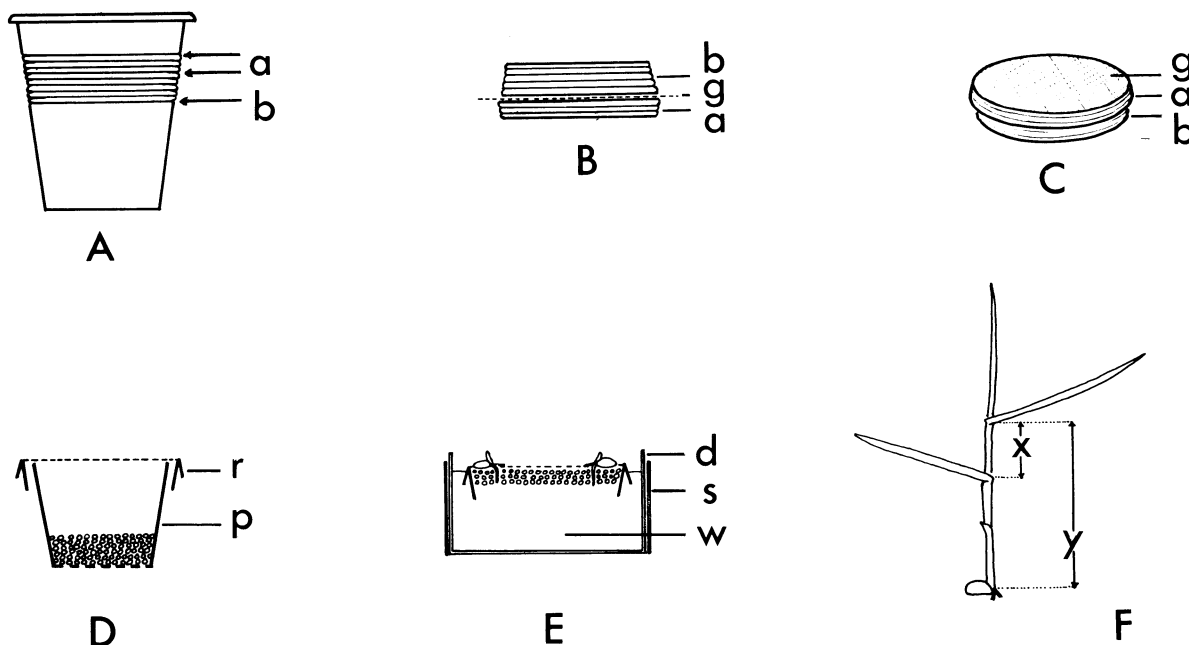


FIGURE 31.— *A, B, C, D, E*, Drawings of equipment used to culture wheat seedlings; *F*, drawing showing methods of taking measurements. (After diagram by H. F. Taylor.)

Suggested standard for comparison—Solutions of (2-chloroethyl)trimethylammonium chloride (CCC) at concentrations of 3×10^{-5} , 10^{-5} , and 3×10^{-6} M.

Reference

Method submitted by H. F. Taylor and B. E. A. Knight, Plant Growth Substance and Systemic Fungicide Unit, Agricultural Research Council, Wye College, Near Ashford, Kent, England.

ROOT GROWTH

AGAR SLANT METHOD

Based on research by R. L. Jones, T. P. Metcalfe, and W. A. Sexton.

Description of method—Seeds of oat and rape are germinated on agar slants containing a test chemical, and the root growth is studied.

This method is also useful in studying the root growth of a wide variety of other kinds of germinating seeds.

Apparatus, Chemicals, and Other Materials

Analytical balance

Test tubes, 1×15 cm.

Test tube racks

Light-proof chamber at room temperature of 21° – 24° C.

About 0.1 g. of each compound to be evaluated

For standard treatment:

250–500 mg. of 2,4-dichlorophenoxyacetic acid

250–500 mg. of isopropyl-N-phenyl-carbamate

Agar

Suggested plant material—Seeds of a crop and a weed plant, such as oat (*Avena sativa* L.) and rape (*Brassica napus* L.).

Preparation and selection of plant material—Sort the seeds, eliminating any that are shriveled or diseased.

Procedure—Prepare about 4% agar in water in an amount sufficient to provide 500 ml. of agar mixture for each concentration of each chemical to be evaluated plus 1,500 ml. to be utilized for an untreated control and for standard treatments with 2,4-dichlorophenoxyacetic acid and isopropyl-N-phenylcarbamate. Dissolve 50 mg. of each test compound in 500-ml. portions of the melted agar mixture.

Pour the agar-water and agar-water-chemical mixture into separate, labeled test tubes, 10 ml. per tube. Slant the tubes so the agar extends about 13 cm. up the side of the tube and allow the agar to solidify with the tube

held in this position. Approximately 46 tubes of each agar-water-chemical medium plus an equal number of plain agar-water tubes will then be available for testing. Use at least 12 replicates of each treatment for statistically reliable results when high concentrations of the chemicals, such as 50 and 100 p.p.m., are employed. Eighteen replicates are sufficient for lower concentrations.

Using half the test tubes of each treatment, place 2 oat seeds on the agar near the top of the slant in each tube. Removal of the husks (glumes) facilitates germination. Place 3 rape seeds on the agar near the top of the slant of each of the remaining test tubes. It may be necessary to apply a slight pressure to partially embed the seeds in the agar to hold them in place.

Place the tubes upright and store them in racks in a dark chamber at room temperature of 21° – 24° C. for the duration of the test. Maintain a high relative humidity in the storage chamber to prevent the agar from drying out and to favor uniform growth of the plants. Drying of the agar must be avoided in order to prevent a corresponding increase in concentration of the test chemical.

Method of taking results—After 5–7 days, or when the rape roots in the untreated control tubes are 5–6 cm. long, measure and record the lengths of the rape roots in each tube. Eight to 10 days after initiation of the test, or when the oat roots in the untreated control tubes are 6–7 cm. long, measure and record the lengths of the oat roots in each tube. Also record the number of seeds in each tube that failed to germinate.

Calculate the average length of the roots in the control tubes, but do not include the zero root growth of the non-germinated seeds in the calculation of these averages. Determine the percentage of growth that the roots of each plant in each treated tube exhibit as compared with the average root length of the untreated control plants.

If one-half or more of the test plants of one species exhibit 50% or greater reduction in root growth in a treatment, the applied chemical is considered to be *very active* under the conditions of the test. If one-half or more of the

test plants of one species exhibit 20–50% reduction in root growth in a treatment, the applied chemical is considered to be *active* under the conditions of the test. If less than one-half of the test plants of one species exhibit greater than 20% reduction in root growth in a treatment, the applied chemical is considered to be *inactive* under the conditions of the test. If a compound is shown to be active or very active at the initial concentrations tested, conduct tests at lower concentrations and repeat until the minimum dosage required to produce an effect classified as *active* is determined.

Suggested standard for comparison—Use 2,4-dichlorophenoxyacetic acid at a concentration of 0.1 p.p.m. as a standard for rape, and isopropyl-N-phenylcarbamate at 0.1 p.p.m. as a standard for oat. 2,4-dichlorophenoxyacetic acid and isopropyl-N-phenylcarbamate at 0.1 p.p.m. are rated as *very active* in this test when applied to rape and oat plants as described.

Reference

Jones, R. L., Metcalfe, T. P., and Sexton, W. A. The Relationship Between the Constitution and the Effect of Chemical Compounds on Plant Growth. I. 2-Phenoxyethylamine Derivatives. *Biochem. Jour.* 45: 143–149. 1949.

CONE CONTAINER METHOD

Based on research by T. K. Pavlychenko.

Description of method—Seeds of barley or other grass plants are dipped in dilute concentrations of a regulating chemical and germinated in soil held in cone-shaped containers. Growth of roots and other parts of the plants is studied in detail by removing the containers and washing away the soil from some of the plants at intervals as the plants mature.

This method can be modified to study the effect of application of different regulating chemicals to either the tops of the plants or to the soil in which the plants are grown.

Apparatus, Chemicals, and Other Materials

Cone-shaped galvanized iron cylinders, 20 cm. diameter at top, 25 cm. at bottom, 61 cm. or more high and fitted with perforated wooden or metal removable bottoms
Analytical balance
Sandy loam soil free from undecomposed organic material, uniformly mixed and well packed into cone-shaped cylinders
Greenhouse maintained at about 18°–27° C.

Beakers or other containers in which to soak the seed

50–100 mg. of each chemical to be evaluated for root growth effects

1–5 g. alpha-naphthaleneacetamide for standard treatment

About 100 ml. 95% ethanol

Suggested plant material—Barley seeds (*Hordeum vulgare* L.), well matured (stored for 8–9 months after harvest), or other grass seeds.

Preparation and selection of plant material—Discard any diseased, broken, or malformed seeds.

Procedure—Dip a number of seeds in aqueous mixtures of each plant regulator at each of 3 dosage levels: 0.002, 0.004, and 0.006% are suggested. The chemicals are dispersed in water with the aid of a small amount of ethanol. The alcohol concentration in the final mixture should not exceed 0.1%. Plant 3 seeds in duplicate cylinders for each chemical and each concentration level. Repeat to obtain duplicate sets that can be examined after 3 wk., 6 wk., and at maturity. Three days after emergence, remove all but the one most vigorous plant in each cylinder. Grow plants in a cool (20°–21° C.) greenhouse for 2 wk.; then increase the temperature to 24°–29° C. thereafter. Dip seeds in water only and grow them in the same manner as for controls.

Method of taking results—Record time of emergence and the height of the plants at 3-day intervals. Harvest a duplicate lot of plants from each treatment and also from the controls at the end of 3 wk., 6 wk., and at maturity, tapping the metal cylinders and then lifting them off over the tops of the plants. Separate roots from tops and record the number of roots, fresh and dry weight of leaves and stems, and the number of tillers. Carefully remove the roots from the soil by immersing the soil together with the roots in water. Gently wash the soil away from the roots with a stream of water. Record depth of root penetration, number and length of roots, and then the dry weight. Compare all of these measurements with those of the controls.

Suggested standard for comparison—Seeds soaked in 0.002, 0.004, and 0.006% alpha-naphthaleneacetamide.

Reference

Unpublished method proposed by T. K. Pavlychenko, Amchem Products, Inc., Ambler, Pa.

IMPREGNATED FILTER PAPER METHOD

Based on research by J. W. Brown; Vivian K. Toole, W. K. Bailey, and E. H. Toole.

Description of method—Root growth of germinating seeds on filter paper treated with the test chemicals is observed.

Apparatus, Chemicals, and Other Materials

Analytical balance
 Pressure-sensitive tape with adhesive on both sides, Scotch brand No. 400, 13 mm. wide
 Filter paper, Whatman No. 1, 8 × 23 or 8 × 46 cm., depending on seed size
 Filter paper strips 1 cm. wide with 6-mm. holes punched with their centers 1–3 cm. apart. For large seeds such as bean, pea, sunflower, and corn use holes 3 cm. apart; for small seeds, 1 cm. apart
 Paper drinking cups, 10 cm. tall
 Metal trays 48 cm. wide × 56 cm. long × 15 cm. deep with false perforated bottoms 3 cm. above the solid bottom of the tray
 Glass plates large enough to cover completely the top of the metal trays
 Constant-temperature chamber maintained at about 28° C.
 10 mg. of each compound to be evaluated
 0.5–1 g. of 2,4-dichlorophenoxyacetic acid for standard treatment.
 Acetone

Suggested plant material—Wide variety of seeds—morning-glory (*Ipomoea* spp.), sunflower (*Helianthus annuus* L.), pigweed (*Amaranthus* spp.), crabgrass (*Digitaria* spp.), mustard (*Brassica* spp.), wheat (*Triticum aestivum* L., also called *T. sativum* Lam. and *T. vulgare* Vill.), pea (*Pisum sativum* L.), bean (*Phaseolus vulgaris* L.), buckwheat (*Fagopyrum esculentum* Moench), cotton (*Gossypium hirsutum* L.), cucumber (*Cucumis sativus* L.), barley (*Hordeum vulgare* L.), corn (*Zea mays* L.), oat (*Avena sativa* L.), rice (*Oryza sativa* L.), and rye (*Secale cereale* L.) have been used.

Preparation and selection of plant material—Sort the various kinds of seeds, eliminating any that are shriveled or diseased.

Procedure—Dissolve 10 mg. of the test chemical in 5 ml. of 100% acetone; then dilute to 1 liter with tapwater, making a 10-p.p.m. solu-

tion. Make 1- and 0.1-p.p.m. solutions by diluting aliquots of this original solution with a 0.5% concentration of acetone in water.

Place the pressure-sensitive tape along one edge of the large piece of filter paper, extending the tape 1 cm. past the edge of the large paper. Place the filter paper strip with the evenly spaced holes on top of the tape. Make the length of paper strip such that it will leave fifteen 6-mm. disks of adhesive exposed. Place 15 seeds of the first species to be tested on the exposed disks, one seed to a disk. Roll the filter paper with the seeds inside and hold the roll in place with the exposed pressure-sensitive tape. Repeat using additional species.

Stand the rolls upright in the paper cups with the part containing the seeds uppermost. Pour 70 ml. of a concentration of one chemical over the rolls containing seeds of each plant species to be tested. Repeat with other concentrations and chemicals. On one set of rolls containing seeds pour an equal volume of a 0.5% concentration of acetone in water to serve as controls.

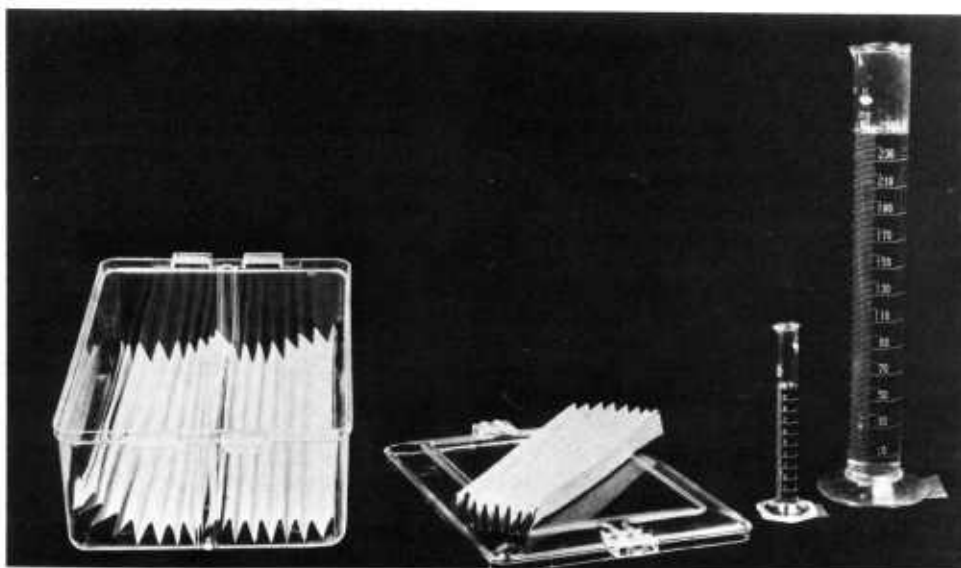
Place the cups containing the treated and the control rolls in the metal trays filled with water to a depth of 1 cm. beneath the perforated false bottom. Cover the trays with the glass plates to maintain a high relative humidity, and place the trays in a chamber maintained at about 28° C.

Large seeds such as peanuts require a relatively large quantity of water to germinate and they are sensitive to submergence in a liquid. For these the following procedure is suggested. Line plastic boxes about 20 × 25 × 10 cm. with paper toweling or filter paper, using 2 thicknesses of the paper (25 × 100 cm.) folded to form corrugations 2 cm. high (fig. 32). Wet the paper with 260 ml. of the test solution and place the seeds (100 per box) between the grooves of the paper with the radicle end down. Place covers on the boxes. Temperatures, solutions, and method of taking results are the same as those of the method described more fully here.

Method of taking results—After 72–96 hr. (depending upon the germination rate of the species), open the seed rolls, and measure the length of the primary roots to the nearest centimeter. The average length of the 10 longest roots is used for computing the percent inhibition of root elongation:

Average control root length
 (acetone-water solution) minus
 average treated root length

————— × 100 = percent inhibition
 Average control root length
 (acetone-water solution)



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FIGURE 32.—Equipment used to germinate large seeds.

If the root elongation is obviously affected by some factor other than the test compound, such as profuse fungal or bacterial contamination, or if the control roots average less than 5 cm. long, rerun the test.

Suggested standard for comparison—1 p.p.m. of 2,4-dichlorophenoxyacetic acid in tapwater containing a concentration of 0.5% acetone.

References

- Brown, J. W. Two Tests for Herbicide Screening. North-eastern Weed Control Conference Proc. (Sup.) 7: 71-74. 1953.
 Toole, V. K., Bailey, W. K., and Toole, E. H. Factors Influencing Germination of Peanut Seeds. Plant Physiol. 39: 822-832. 1964.

NUTRIENT MIST METHOD

Based on research by B. T. P. Barker; W. Carter; L. J. Klotz; M. C. Vyvyan and G. F. Trowell.

Description of method—Trees are grown with their roots in a closed chamber containing air filled with a fine mist of nutrient solution in which the chemical being tested has been dissolved or suspended, and effects of the regulating chemical on growth of the roots are observed.

This method can be adapted to study growth responses of many different kinds of plants.

Apparatus, Chemicals, and Other Materials

Mist chamber⁴
 Analytical balance
 5 g. of each compound to be evaluated
 Nutrient solution
 Optional materials:
 lanolin
 solvent, such as 95% ethanol
 wetting agent, such as Tween 20

Suggested plant material—Young trees—maple (*Acer* spp.), pine (*Pinus* spp.), oak (*Quercus* spp.), apple (*Malus sylvestris* Mill., also called *Pyrus malus* L.), peach (*Prunus persica* [L.] Batsch), and *Citrus* spp.

Preparation and selection of plant material—Select young trees at a uniform stage of development and growing at approximately the same rate.

Procedure—Support trees (2-6 of each species) so their roots are suspended in the chamber containing air filled with a mist of nutrient solution. Set up duplicate groups of trees for each additional compound or concentration to be tested. Add the plant regulator or other chemical to be tested directly to the feed tank containing the nutrient solution (concentrations of 0.01-10 p.p.m. suggested). Maintain

⁴ Construction of various types of mist chambers is described in articles listed at the end of this method.

comparable sets of trees with their roots in the nutrient mist only and designate these as controls.

As an alternative method, the chemical to be tested can be applied to the exposed tops of the trees as overall aqueous sprays (1–1,000 p.p.m. suggested) or in a lanolin-paste mixture as a narrow band on the stem (100–10,000 p.p.m. suggested).

If regulating chemicals are to be applied uniformly to all the trees, several trees may be grown in the same chamber. The size of the chamber may be varied from approximately 1-liter capacity to room size.

Method of taking results—After roots of the controls have developed a measurable amount of new growth, measure and record the length

of all of the roots, and record the number of those living and dead on both treated and control trees.

Suggested standard for comparison—No standard treatment suggested.

References

- Barker, B. T. P. Studies of Root Development. Long Ashton Res. Sta. Ann. Rpt. 1921: 9–20.
 Carter, W. A Method of Growing Plants in Water Vapor to Facilitate Examination of Roots. *Phytopathology* 32: 623–625, 1942.
 Klotz, L. J. A Simplified Method of Growing Plants with Roots in Nutrient Vapors. *Phytopathology* 34: 507–508, 1944.
 Vyvyan, M. C., and Trowell, G. F. A Method of Growing Trees with Their Roots in a Nutrient Mist. East Malling Res. Sta. Ann. Rpt. 1952: 95–98, 1953.

ROOT INDUCTION

ROOTING COFACTOR METHOD

Based on research by C. E. Hess; variation by S. Challenger, H. J. Lacey, and B. H. Howard.

Description of method—A bioassay designed to detect naturally occurring substances that stimulate root initiation in the presence of indole-3-acetic acid. Extracts of easy-to-root plants, such as the juvenile form of English ivy (*Hedera helix*, L.) and *Chrysanthemum*, contain 4 chromatographically separable substances that stimulate root initiation of mung bean cuttings in the presence of indole-3-acetic acid. An increase in the number of roots per cutting over the controls (cuttings with indole-3-acetic acid alone) indicates the presence of a rooting cofactor. A decrease in the number of roots is an indication of the presence of an inhibitor.

Apparatus, Chemicals, and Other Materials

Vermiculite
 Porcelain pan, 43 × 30 × 6 cm.
 Growth chamber maintained at 27° C., 60% relative humidity, and with about 3,000-foot-candle light intensity from a combination of fluorescent and incandescent lamps; 16-hr. photoperiod. A greenhouse can be used but the results will be variable because of variations in light intensity.
 Shell vials, 19 × 65 mm.
 Razor blade
 Water aspirator
 Waring Blender

Separatory funnel

Chromatographic equipment (ascending or descending)

Chromatographic paper, Whatman No. 3 MM

Lyophilizer

pH meter

Ether

Chloroform

About 500 mg. of indole-3-acetic acid and an equal amount of catechol for standard treatment

Methanol

Ethanol

Isopropanol

Distilled water

Sodium hypochlorite

Suggested plant material—Nine- to 10-day-old seedlings of mung bean (*Phaseolus aureus* Roxb.—large oriental variety).

Preparation and selection of plant material—Treat 95–100 ml. of dry mung bean seeds for 3 minutes in a 0.33% solution of sodium hypochlorite. Rinse the seeds and then soak them in running tapwater for 18–24 hr. Place the seeds on moist vermiculite in a porcelain pan and cover them with a layer of moist vermiculite about 1.5 cm. thick. Germinate the seeds in a growth chamber.

Make cuttings from selected, uniform seedlings 9–10 days old. Do this when the primary leaf is fully expanded and the trifoliate leaf bud has not expanded. Prepare the cuttings by

cutting off the seedling root system and removing any cotyledons that have not abscised. The cutting consists of 3 cm. of hypocotyl, plus the epicotyl, the primary leaves, and the unexpanded trifoliate leaf bud. Select cuttings of uniform size and shape to avoid wide variations between individual cuttings in the same vial.

Procedure—Place 5 or 10 cuttings in each of 17 shell vials, together with 4 ml. of a 5×10^{-6} M solution of indole-3-acetic acid per vial. The incubation solution will be taken up by the cuttings within 18–24 hr. Whenever required, replace with distilled water the amount of solution taken up during the 6- to 7-day period allowed for root growth.

To obtain substances to use in this rooting test, extract lyophilized tissue or fresh tissue. If lyophilized tissue is used, extract 50–200 mg. of ground, lyophilized tissue 3 times with 25- to 100-ml. portions of absolute methanol. Combine the extracts and concentrate them under reduced pressure, using a water aspirator to provide the vacuum. Streak the extracts across a strip of chromatographic paper 5 cm. wide. Develop the chromatogram (descending) with isopropanol-water (8:2 v/v) after a 3-hr. period for equilibration. Allow the solvent to descend 30 cm. below the origin.

If fresh tissue is used, boil it in ethanol or methanol for at least 1 minute, and then grind it in a Waring Blendor. Filter and extract the residue 2 more times with fresh portions of ethanol or methanol. Combine the extracts and evaporate to dryness. Dissolve the extract in 80% ethanol and streak the solution on the chromatographic paper. Chromatograph as described above.

After drying the chromatogram, divide it into fifteen 2-cm. segments. Fold each piece (2×5 cm.) and place each separately in a shell vial. For controls, cut from the same chromatogram 2 comparable pieces of paper, i.e., one 2×5 -cm. piece from above the origin, and one piece of this size from below the solvent front. When each segment is in a corresponding vial, add 4 ml. of a 5×10^{-6} M solution of indole-3-acetic acid to each vial. Place the cuttings in the vials as described above and return them to the growth chamber.

In addition to separation by paper chromatography, preliminary fractionation is possible. Evaporate the extract. Dissolve the extract by shaking it, together with chloroform and water, in a separatory funnel. The chloroform layer will contain Cofactor 4. Acidify the remaining aqueous layer to a pH of 3.0 and partition it with ether. Cofactor 3 will go into the

ether layer, and Cofactors 1 and 2 will remain in the aqueous layer.

Method of taking results—Count the number of roots on each cutting 6–7 days after the cuttings are made. Compare the average number of roots per cutting for each treated segment with the average number of roots per cutting for the control segments. Prepare a histogram to show peaks of promotion and inhibition.

The approximate Rf values of the 4 rooting cofactors using the isopropanol-water solvent system are:

	<i>Rf value</i>
Cofactor 1	0.1
Cofactor 2	0.3
Cofactor 3	0.6
Cofactor 4	0.8

Suggested standard for comparison—Indole-3-acetic acid 5×10^{-6} M; indole-3-acetic acid 5×10^{-6} M together with catechol 3×10^{-4} M; and catechol 3×10^{-4} M. The combination of indole-3-acetic acid and catechol is normally synergistic.

Variation used by Challenger, Lacey, and Howard—To prepare plant extracts for use in testing for root cofactors, cut 3-cm. segments from stems of the plants to be tested. Place these, basal end downward, in moist sand for a period sufficient to allow callus to develop or root primordia to become apparent. Remove the cuttings, wash the sand from the basal ends, and then immerse the cuttings for 24 hr. in 96% methanol at 3° C. Collect the extract and repeat the extraction for another 24 hr. Combine the 2 extracts and evaporate the alcohol under vacuum at 25° C. Centrifuge the aqueous residue. Extract the water-soluble materials with ether, first at pH 8 and then at pH 4. Apply replicate aliquots of the acid fraction to strips of Whatman No. 1 filter paper and develop, using descending chromatograms and isopropanol-water-ammonia (10:1:1 v/v) as a solvent system. Dry the strips in darkness and divide each strip into 10 segments. Determine the amount of activity in each segment by means of the rooting cofactor bioassay.

References

- Challenger, S., Lacey, H. J., and Howard, B. H. The Demonstration of Root Promoting Substances in Apple and Plum Rootstocks. Ann. Report of the East Malling Res. Sta. 1964: 124–128. 1965.
- Hess, C. E. A Physiological Analysis of Root Initiation in Easy and Difficult-to-Root Cuttings. XVI Internat. Hort. Cong. Proc. 4: 375–381. 1964.
- Hess, C. E. Naturally Occurring Substances Which Stimulate Root Initiation. Régulateurs Naturels de la Croissance Végétale, Cent. Natl. de la Rech. Sci., Paris, 123: 517–527. 1964.

ROOT PRIMORDIA METHOD

Based on research by Tetsuo Takematsu.

Description of method—The number of root primordia induced on young radish stems (hypocotyls) depends on the kind and concentration of growth substance applied. Roots and hypocotyls of intact radish seedlings are placed for a specified length of time in a solution of the test compound and then transferred to sand. The number of induced root primordia that form on hypocotyls of treated plants is compared with the number of primordia that form on hypocotyls of the control plants. The difference represents the root-inducing capacity of the compound.

Apparatus, Chemicals, and Other Materials

- Petri dishes, large
- Sand
- Analytical balance
- Glass cylinder (jar or vial), 3.5 cm. deep and 3 cm. in diameter
- Transparent glass plate
- Black paper
- Dissecting needle
- Tweezers
- Magnifying glass or microscope of low magnitude
- 10 mg. of each compound to be evaluated
- 10 mg. of indole-3-acetic acid or alpha-naphthaleneacetic acid, and 10 mg. of 2,4-dichlorophenoxyacetic acid for standard treatments

Suggested plant material—Hypocotyls of young radish plants (*Raphanus sativus* L.)—Riso Daikon variety, or other varieties with a high degree of sensitivity to root-inducing compounds.

Preparation and selection of plant material—Plant radish seeds 0.5 cm. deep in the large petri dishes two-thirds filled with sand and maintain the moisture content of the sand at a level of 20% water by weight. Place the dishes in diffused light at a temperature of about 24° C. Three to 4 days after germination, add sufficient water to the dishes so seedlings can be pulled easily from the sand without damaging the roots or hypocotyls. Select seedlings of uniform size and shape with well-formed cotyledons, with hypocotyls approximately 3–4 cm. long, and with taproots of approximately the same length. Keep the selected plants temporarily in petri dishes containing water to pre-

vent wilting. It is not necessary to wash the sand from the roots.

Procedure—Prepare a series of dilutions of the growth substances to be tested by dissolving each regulating compound in a small amount of alcohol (not to exceed 0.1% of the water on a volume basis). Then, while stirring, add the alcoholic solution to the required amount of water at concentrations from 0.01–500 p.p.m. Place each concentration in a separate glass cylinder to two-thirds of the cylinder's depth. Soak roots and hypocotyls of each group of 15–20 radish plants in respective test solutions contained in the glass cylinders. The duration of the treatment varies according to the growth substances to be tested and their concentrations. For example, use a 12- to 24-hr. period for 5-p.p.m. solution of indole-3-acetic acid, 5-p.p.m. solution of alpha-naphthaleneacetic acid, and 0.5- to 1-p.p.m. solution of 2,4-dichlorophenoxyacetic acid. For comparison, place one lot of plants into a cylinder containing distilled water for a corresponding period.

Transplant the treated and untreated seedlings from cylinders to the large petri dishes containing sand maintained at a moisture level of 25% and keep them in a room at 70–80% humidity and about 20°–24° C.

Method of taking results—Seventy-two hours after the treated plants and the controls are placed in the sand, remove each plant from the petri dishes and wash the sand from the roots. Discard wilted plants. Place the plants on a transparent glass plate backed with black paper. Strip off the cortex of the hypocotyls with a dissecting needle and tweezers, exposing the central cylinder (stele). Examine the stele of each hypocotyl with a magnifying glass or microscope and record the number of root primordia. The number of primordia on the treated hypocotyls compared with the number on the hypocotyls kept in distilled water indicates the effectiveness of the growth-regulating substance as a root-inducing compound. Measure 10–20 plants to obtain the average root primordia formation for each lot of seedlings.

Suggested standard for comparison—5 p.p.m. of indole-3-acetic or alpha-naphthaleneacetic acid solutions and 0.5–1 p.p.m. solution of 2,4-dichlorophenoxyacetic acid for 12–24 hr.

Reference

Takematsu, Tetsuo. New Methods for the Qualitative and Quantitative Assays of Growth Substances; Research on the *Raphanus* Test and Its Application. Utsunomiya Univ., Col. of Agr. Spec. Bul. 12, 193 pp. 1961.

STEM-CUTTING METHOD

Based on research by J. W. Mitchell and P. C. Marth; H. B. Tukey; J. S. Wells.

Description of method—Cuttings are soaked, dusted, or dipped in mixtures containing chemicals, and the effect on root initiation and growth is determined.

Compounds that affect top growth can also be applied separately or simultaneously with root-inducing substances.

Apparatus, Chemicals, and Other Materials

Knife with sharp, thin blade 10–15 cm. long
Analytical balance

Containers of suitable size to hold cuttings upright in solutions

Propagating case with top or sides made of translucent material, with temperature controlled at 18°–21° C. and humidity controlled at 75–95%

Perlite or fine sand washed free of organic matter, clay, or other foreign material

Clay pots, 8 cm. in diameter

Composted soil

100–200 mg. of each compound to be evaluated

500 mg. of 3-indolebutyric acid or alpha-naphthaleneacetamide for standard treatment

Solvent such as 95% ethanol

0.5 kg. of dust carrier such as talc, fuller's earth, or finely ground clay

Suggested plant material—Pieces of stems of woody plants—privet (*Ligustrum* spp.), yew (*Taxus* spp.), osmanthus (*Osmanthus* spp.), and holly (*Ilex* spp.); or of herbaceous plants—chrysanthemum (*Chrysanthemum* spp.), geranium (*Geranium* spp.), lantana (*Lantana* spp.), and carnation (*Dianthus caryophyllus* L.).

Preparation and selection of plant material—**Cuttings from woody plants:** Select terminal shoots during late summer or early fall, or a few weeks after vegetative growth has ceased. The leaves should be fully developed and the wood hard. Plant species vary with regard to the best time of year that cuttings should be taken in order to obtain optimum results. Remove the cuttings from the plants with a sharp knife, making one cut on a slant and at the base of the current season's growth. Cuttings 10–15 cm. long or pieces including 3–6 nodes are then made from these branches. Make the basal cut immediately below a node and the uppermost cut just above a node. Remove the

lower leaves, but let 3 or 4 of the uppermost leaves remain attached. Using rubber bands, fasten cuttings in bundles of 10–20 with stems parallel and bases even. Do not allow cuttings to dry out or the leaves to wilt at any time during these preparations.

Cuttings from herbaceous plants: Stock plants may be maintained under greenhouse or outdoor conditions so that succulent new shoots are available for cutting material throughout the year. Prepare bundles of cuttings for treatment in the same manner as with woody plant material.

Procedure—Three methods of treating cuttings have proved suitable for this test:

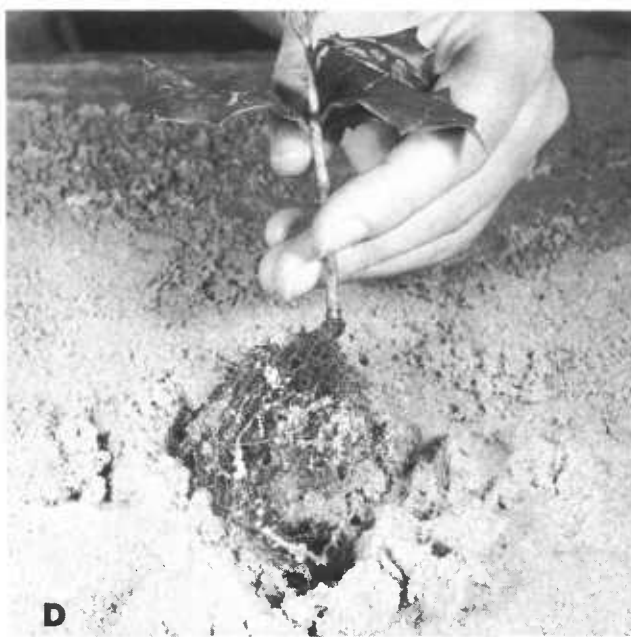
Soak method: Prepare aqueous solutions of each compound to be tested in concentrations of 25, 50, and 100 p.p.m. Stand a bundle of cuttings in each dilution so that the bases of the cuttings are covered with the solution to a depth of about 3 cm. (fig. 33, A). Vary the size of the container to suit the size of the batch of cuttings, and do not allow the leaves to come in contact with the solution. After 3–6 hr. in diffused light, remove the cuttings and insert the basal ends of the stems to a depth of 5–8 cm. in the rooting medium contained within the propagating chamber (fig. 33, B). Pack the moist rooting medium firmly around each stem and water thoroughly. Keep the medium moistened throughout the test.

Dust method: Succulent cuttings are usually treated with a mixture of 1 part of regulating chemical to 1,000–5,000 parts of dust carrier; woody cuttings usually require a stronger dust mixture—1 part regulator in 500–750 parts of dust carrier. To prepare dust mixtures, first dissolve the desired weighed amount of the compound in a volatile solvent such as ethanol, then add the proportionate weighed amount of the dust carrier. The volume of solvent should be sufficient to make a thin paste of the regulator-dust mixture. Stir the entire mixture thoroughly; then allow the solvent to evaporate at room temperature, with occasional stirring during this process.

Treat each cutting individually by rolling about 3 cm. of the basal part in the dust mixture so that a thin coating of dust adheres to it. If the dust fails to adhere, moisten the end of the cutting before applying the dust (fig. 33, C). Tap off any loose dust and set the treated portion of the cutting in the rooting medium (fig. 33, B). Do not rub the dust from the cuttings when inserting them in the medium; place in a trench wide enough so the dust remains on the stem.

Dip method: Prepare a solution of each regulating chemical, using 50-95% ethanol. Vary the concentration of each compound in a range of 100-500 p.p.m. for succulent cuttings and

500-1,000 p.p.m. for woody cuttings. Dip the bases of one bundle of cuttings to a depth of 3 cm. in one dilution of a compound, and insert the treated portion of these cuttings individ-



PN-298, A, B, C, and D

FIGURE 33.—Applying regulating chemicals to determine their effect on initiation of roots by stem cuttings: A, Soaking cuttings in solution of test compound; B, inserting cuttings in rooting medium; C, dusting cuttings with a mixture of test compound; D, examining cuttings to determine whether roots have formed.

ually in the rooting medium. Repeat, using other bundles of cuttings for each concentration of each test chemical.

Method of taking results—Examine several of the cuttings at intervals to determine whether roots have formed (fig. 33, *D*).

After 10–14 days in the case of succulent cuttings, or 14–30 days in the case of woody ones, carefully loosen the rooting medium around the cuttings and remove each one individually from the medium. Sort them into the following categories: (a) Those that are dead, (b) those not rooted, (c) those with a few short roots, (d) those with a moderate number and fairly long roots, (e) those with many relatively thick, long roots. Note and re-

cord the number of cuttings in each category. Also note any unusual characteristics of roots in each category. Pot the cuttings, using soil, and observe their subsequent growth.

Suggested standard for comparison—Soak method, using 3 concentrations of 3-indolebutyric acid or alpha-naphthaleneacetamide (25, 50, and 100 p.p.m.) for 2–4 hr.

References

- Mitchell, J. W., and Marth, P. C. *Growth Regulators*. 129 pp. Univ. of Chicago Press, Chicago, Ill. 1947.
 Tukey, H. B., editor. *Plant Regulators in Agriculture*. 269 pp. John Wiley and Sons, Inc., New York. 1954.
 Wells, J. S. *Plant Propagation Practices*. 344 pp. Macmillan Co., New York. 1955.

SEED FORMATION

LANOLIN PASTE METHOD

Based on research by S. L. Emsweller and N. W. Stuart; R. E. Wester and P. C. Marth.

Description of method—A mixture of lanolin and a regulator is applied to a wound on the petal base or on the pedicel, and the effect on seed development is observed.

Apparatus, Chemicals, and Other Materials

Clay pots, 15–25 cm.

Composted soil

Space in greenhouse maintained at about 21°–29° C. by day and 18°–21° C. by night

Small vials, 25–50 ml., or other containers

Analytical balance

250–500 mg. of each compound to be evaluated

1 g. each of alpha-naphthaleneacetamide, para-chlorophenoxyacetic acid, and 3-indolebutyric acid for standard treatment

Lanolin

Tween 20

Suggested plant material—Species of *Lilium* such as *L. longiflorum* Thunb, (Creole, Croft, or Ace clones); bush lima beans (*Phaseolus lunatus* L.), Fordhook, Early Market, or Peerless varieties; or other plants difficult to hybridize.

Preparation and selection of plant material—Grow the plants, known to be difficult to cross, under usual greenhouse conditions.

Procedure—Prepare a range of concentrations of the chemical to be tested in lanolin, as described on p. 7.

Lilies: Immediately before hand-pollinating the flowers, separate one “petal” (i.e., one member of the outer whorl of the perianth) from the receptacle, and cover the injured part of the receptacle with lanolin-regulator mixture.

Lima beans: Immediately after cross-pollination has been completed, scratch the base of the flower stalk (pedicel) lightly with a pin or dissecting needle. The wound should be made close to the juncture of the pedicel and peduncle. With a pin or dissecting needle, place a small portion of the lanolin-regulator mixture on this wounded surface. Pollinate additional flowers to use as controls.

Method of taking results—Observe and record the number of fruits and their rate of development. When fruits are mature, remove the seeds and record the number of mature and immature seeds. Determine the viability of the seeds.

Suggested standard for comparison—For lima beans, make a lanolin mixture containing sufficient 3-indolebutyric acid to make a concentration of 0.1%, and a second lanolin mixture containing sufficient para-chlorophenoxyacetic acid to make a 0.1% mixture. Combine portions of these two mixtures in a ratio of 4 parts of the 3-indolebutyric mixture to one part of the phenoxyacetic acid mixture.

References

Emsweller, S. L., and Stuart, N. W. Use of Growth-Regulating Substances to Overcome Incompatibilities in *Lilium*. Amer. Soc. Hort. Sci. Proc. 51: 581-589. 1948.

Wester, R. E., and Marth, P. C. Some Effects of a Growth Regulator Mixture in Controlled Cross-Pollination of Lima Bean. Amer. Soc. Hort. Sci. Proc. 53: 315-318. 1949.

DETECTION OF EXOGENOUS GROWTH REGULATORS IN PLANTS

GRAFT METHOD

Based on research by T. J. Muzik and J. W. Whitworth.

Description of method—The presence of a growth-regulating substance or a metabolite of it in a plant previously treated with the chemical is determined qualitatively by grafting a scion from an untreated plant of the same kind to the plant being tested. Malformation of leaves that develop on the scion, typical of those induced by the compound involved, indicates that there is an effective, translocatable amount of the compound or a metabolite of it in the test plant.

Apparatus, Chemicals, and Other Materials

Beaker, 1 liter
Razor blade or scalpel
Composted soil
Clay pots
Analytical balance
0.5 g. of compound to be evaluated
0.5 g. of 2,4-dichlorophenoxyacetic acid for standard treatment

Suggested plant material—Young, vigorously growing tomato plants (*Lycopersicon esculentum* Mill.). Other kinds of plants that can be readily grafted and are sensitive to the growth-regulating chemical involved can also be used.

Preparation and selection of plant material—Grow tomato plants in composted soil maintaining conditions that afford vigorous growth throughout this period. Select 24 plants of uni-

form size about 6-8 wk. old and set aside 8-10 others as a source of scions.

Procedure—While the fourth leaf from the base on each plant of the first group of 24 is still attached, dip each of these leaves into a water solution containing 1,000 p.p.m. of the compound to be evaluated. Excise each of the treated leaves after 8 days. Collect 4 buds or 4 short branches from the untreated plants. Graft one of these on each of the 4 treated plants at the second internode above the excised leaf. Collect additional scions from the untreated plants 2 wk. later and graft them to 4 more of the treated plants. Repeat the grafting procedure at 2-wk. intervals for 60-90 days.

Method of taking results—Allow the scions on all grafts to grow and produce several leaves. Examine these and record the extent to which the new leaves are malformed. Presence of malformed leaves indicates that sufficient growth regulator or a derivative of it was translocated from the treated plants to the scion to influence growth. Determine the length of time after treatment during which positive results are obtained.

Suggested standard for comparison—2,4-dichlorophenoxyacetic acid at concentration of 1,000 p.p.m.

Reference

Muzik, T. J., and Whitworth, J. W. Growth-Regulating Chemicals Persist in Plants: Qualitative Bioassay. Science 140: 1212-1213. 1963.

DETECTION OF EXOGENOUS PLANT GROWTH REGULATORS IN ANIMALS

BEAN STEM METHOD

Based on research by J. W. Mitchell, R. E. Hodgson, and C. F. Gaetjens.

Description of method—Regulating chemicals are extracted from animal tissues, fluids, secretions, or excrement. The extract is applied to stems of young bean plants, and elongation in-

icates the presence of plant-regulating compounds in the extract.

The method described here has been used to estimate the amount of 2,4-dichlorophenoxyacetic acid (or its salts) in the blood of an animal to which this compound was fed. The method has also been used to detect the presence of this chemical in the animal's tissues and milk. Naturally occurring, cell-elongating compounds present in urine and feces can also be detected through the use of this method.

Apparatus, Chemicals, and Other Materials

Millimeter rule

Pipette, 0.1 ml. capacity graduated in 0.01 ml.

Fluorescent lights that will supply 800 foot-candles of light intensity at leaf surface

Wiley Mill or suitable means of grinding samples to 60 mesh

Glass containers for holding samples

Analytical balance

1–2 g. of sodium or ammonium salt of 2,4-dichlorophenoxyacetic acid for standard treatment

Centrifuge

Composted soil

Clay pots, 8 cm.

Suggested plant material—Young bean plants (*Phaseolus vulgaris* L.)—Black Valentine or Pinto variety.

Preparation and selection of plant material—Germinate bean seeds in composted soil contained in 8-cm. clay pots, using several seeds per pot. Allow the plants to grow in a well-illuminated greenhouse until they are about 9 cm. tall. If the temperature is maintained near 27° C. during germination and adequate sunlight is available, the plants will produce sturdy stems and reach the required height in about 5 or 6 days. Select plants having first internodes that do not vary more than 1 mm. in length from the average of the entire group selected. The average internode length of the selected plants should be, whenever possible, 13 mm. but may in some cases be 12 or 14 mm. Select a sufficient number of plants so 10 can be used for each treatment. Arrange the plants under the fluorescent lights, making certain that the lights extend beyond the edge of the area covered by the plants so the plants can obtain light, solely from the artificial source, of relatively uniform intensity throughout. Number each plant, and measure to the nearest mm. the length of each first internode. Assign treatments in the form of randomized blocks.

Procedure—Prepare 4 aqueous solutions of sodium 2,4-dichlorophenoxyacetate, the first containing 60 p.p.m. of the salt (6 mg. of salt in 100 ml. water), the second, third, and fourth containing 30, 15, and 7.5 p.p.m., respectively, of the salt. Make these by diluting the more concentrated solutions with water.

In detecting the regulator in blood, obtain blood serum from animals that have not ingested the chemical under test, and also serum from animals that have been fed the test compound. Measure five 3-ml. portions of the control serum. Prepare standard growth-regulator-serum mixtures by adding none of the aqueous salt solutions to the first portion of control serum, 1 ml. of the 60 p.p.m. solution to the second portion of serum, 1 ml. of the 30 p.p.m. solution to the third portion of serum, continuing until each concentration of salt has been added to a corresponding portion of control serum. Measure several 3-ml. portions of serum from the animals that were fed the growth regulator.

Evaporate separately all the serum samples on a steam bath. Grind the residues quantitatively through a 60-mesh screen and extract each sample for 15 minutes with 5 ml. of hot distilled water. Filter the mixtures and wash the residues with hot water. Evaporate the filtrates on a steam bath just to dryness, cool the receptacles, and wash the residues obtained into centrifuge tubes using 1 ml. of water for each, and centrifuge for 5–10 minutes. Decant the centrifugate and apply 0.01 ml. of each preparation to each of 10 plants. Place the measured serum preparation on the first internode of each plant as a narrow band (approximately 2 mm. wide) extending around the internode midway between the first and second node. Deliver the measured volume directly from the 0.1 ml. pipette onto the stem. Illuminate the plants, using only the artificial light source, with 12 hr. of light, followed by 12 hr. of darkness.

Method of taking results—Twenty-four hours after treatment, measure the length of each first internode to the nearest mm. Calculate the elongation that occurred during the period. Compare the elongation of plants that received serum from untreated animals with that of plants that received serum from treated animals. Determine if the differences are statistically significant. Draw a standard curve showing internode elongation induced by the known amounts of 2,4-dichlorophenoxyacetate when added to serum from the untreated animals. Using this standard curve, express the amount of elongation obtained with serum

from the treated animals in terms of parts per million of 2,4-dichlorophenoxyacetate equivalent.

Use this method for measuring the amount of the salt in animal organs or in milk.

Suggested standard for comparison—Sodium or

ammonium salt of 2,4-dichlorophenoxyacetic acid used as described above.

Reference

Mitchell, J. W., Hodgson, R. E., and Gaetjens, C. F. Tolerance of Farm Animals to Feed Containing 2,4-Dichlorophenoxyacetic Acid. *Jour. Anim. Sci.* 5: 226-233. 1946.

DETECTION OF REGULATORS IN WATER

ROOT GROWTH METHOD

Based on research by D. Ready and V. O. Grant.

Description of method—Small amounts of 2,4-dichlorophenoxyacetic acid in aqueous solutions are detected by the inhibiting effect on growth of primary roots of cucumber.

This test may also be adapted for the detection of other compounds in water solution provided the roots of the test plant used are sufficiently sensitive to the chemical being tested.

Apparatus, Chemicals, and Other Materials

Petri dishes, 15 cm. in diameter
Filter paper
Analytical balance
Darkened constant-temperature room maintained at about 28° C.
1 g. of 2,4-dichlorophenoxyacetic acid for standard treatment
Distilled water

Suggested plant material—Cucumber seeds (*Cucumis sativus* L.).

Preparation and selection of plant material—Use seeds having a high percentage of germination; sort them, and discard those that are defective.

Procedure—Place 15 cucumber seeds on filter paper in the bottom of each petri dish. Using

5 petri dishes for each concentration level of each solution to be tested, moisten the paper in each dish with 15 ml. of the test solution. A range of 15 concentrations varying in uniform steps from 0.001 p.p.m. to 10 p.p.m. is suggested. Cover the petri dishes and place them in a darkened constant-temperature room maintained at about 28° C. Prepare 5 dishes, using distilled water instead of the test solution, and maintain these in the same manner for controls.

Method of taking results—After 96 hr., measure the length of the primary root of each seedling in each dish to the nearest millimeter. Compare these root measurements with those of the control seedlings and the seedlings treated with a known range of concentrations of 2,4-dichlorophenoxyacetic acid. The amount of reduction in length of the primary roots indicates the amount of the plant regulator in the test solution.

Suggested standard for comparison—2,4-Dichlorophenoxyacetic acid at concentrations of 0.001, 0.005, 0.025, 0.050, 0.075, 0.10, 0.25, 0.50, 0.75, 1, 2.5, 5, 7.5, and 10 p.p.m.

Reference

Ready, D., and Grant, V. O. A Rapid Sensitive Method for Determination of Low Concentrations of 2,4-Dichlorophenoxyacetic Acid in Aqueous Solution. *Bot. Gaz.* 99: 39-44. 1947.

PENETRATION OF SOIL BY REGULATORS

SEED METHOD

Based on research by P. J. Linder.

Description of method—Chemicals are applied to the surface of soil contained in glass tubes. Soil samples are collected as thin layers from various depths and bioassayed for the presence of the chemical.

This method is useful in studying the effect of simulated rainfall on the movement of regulating chemicals through soil, the effect of various adjuvants, types of soil, and the physical structure of the soil on the rate of movement of these compounds through soil. This method also can be adapted to the study of persistence of regulating chemicals in soil as affected by fac-

tors such as temperature, moisture, duration of exposure, and microbial content of the soil.

Apparatus, Chemicals, and Other Materials

Straight glass tubes, 3 cm. in diameter \times 14 cm. long

Cork stoppers to plug one end of the glass tubes

Fertile soil passed through a screen with 0.6-cm. openings

Cork stoppers with a maximum diameter slightly less than the inside diameter of the glass tubes

Dowel, 0.6 cm. in diameter, 30 cm. long

Analytical balance

Hand sprayer capable of distributing a test solution accurately over a given area

Razor blade

Shallow tin or plastic cups or screw-type jar lids, about 6 cm. diameter

Dark chamber maintained at about 27° C. with a high relative humidity

About 5 g. of each compound to be tested

Suggested plant material—Seeds of mustard (*Brassica* spp.) or other small-seeded plants known to be sensitive to the compounds to be tested.

Preparation and selection of plant material—Sort the seeds, eliminating any that are shrunken or defective.

Procedure—Tightly stopper one end of the glass tube. Pour 75 g. of air-dry soil into the stoppered tube. Then place a loose-fitting cork on top of the soil. Place the end of the dowel against the loose-fitting cork and subject the other end of the dowel to a pressure of 2 kg. to compress the soil a measured amount.

Remove the tightly fitting cork stopper from the tube and, by pressing the dowel against the loose-fitting stopper, force the soil upward in the tube until the surface is flush with the open end of the tube. Mark off an area of about 1 sq. m. on the floor. If necessary, cover the area with disposable paper or other material. Stand the tubes to be used for one treatment on end within the marked area so the flush and exposed soil surfaces are uppermost.

Apply the compound to be evaluated evenly over the entire marked area including the exposed soil surfaces. This can be done by spraying the mixture back and forth over the entire area, first in one direction, and then in a direction at right angles to this, so the area is covered uniformly with the mixture.

Using separate sets of tubes of soil, various amounts of the chemical can be tested. To de-

termine the amount of each chemical to be used, carry out preliminary experiments with each compound to find the dosage range necessary to inhibit shoot extension above the soil from 0–100%. Use 10 soil tubes for each concentration of each chemical, and treat 5 tubes with only water and solvent, if a solvent was used, to serve as solvent-treated controls.

To test for the presence of the applied compound, collect a soil sample by using the dowel and loosely fitting stopper to push the column out through the open end of the cylinder for a distance of 3 mm.; then slice off the ejected portion with a razor blade. In this way it is possible to collect a surface layer 3 mm. or more in thickness, or any lower layer of soil desired.

To obtain a sufficient volume of soil for assaying, collect comparable samples from 5 replicates and combine and mix them. Divide the composite sample into 3 equal parts. Place each soil sample in the shallow tin or plastic cup. Sow seeds of the test plants uniformly in the soil. Add uniformly sufficient water to bring each soil sample to optimum moisture content for seed germination. Store the cups containing the soil and seeds in a dark, moist place at about 27° C.

Method of taking results—Measure the height (or fresh weight) of the plants in the various soil samples, including the prepared standards and the solvent-treated control. Compare the average amount of growth with the growth of solvent-treated controls and with that of the plants grown in cups containing soil samples to which have been applied known concentrations of the test chemical.

The soil in the tubes can be (1) assayed, as described, shortly after chemical application to determine the rate and depth of penetration of the chemical, or (2) stored under controlled moisture, temperature, and other conditions before being assayed to determine the effect of these on persistence of the chemical, or (3) subjected to simulated rainfall for leaching studies, etc., before the assay. The prepared series of standards may be used also to determine the amount of chemical present in field plots that have been treated with a test chemical.

Suggested standard for comparison—No standard treatment suggested.

Reference

Linder, P. J. Movement and Persistence of Herbicides Following Their Application to the Soil Surface. Northeast. States Weed Control Conf. Proc. 6: 7–11. 1952.

RESIDUE OF REGULATORS IN SOIL

SUCCESSIVE CROP METHOD

Based on research by A. J. Loustalot and R. Ferrer; W. W. Robbins, A. S. Crafts, and R. N. Raynor.

Description of method—Several successive crops of plants are grown in soil treated once with a chemical in order to determine how long the chemical remains in the soil in sufficient amounts to affect plant growth.

This test is convenient for testing the effect of cultivation or mixing of the chemical with the surface or with lower layers of the soil, and for studying the effect of environment on the persistence of the chemical in soil.

Apparatus, Chemicals, and Other Materials

Soil to be tested

Unperforated small containers (Clean, used No. 2 tin cans have the advantage over clay pots of not absorbing any of the applied chemicals.)

Analytical balance

Greenhouse space

About 2–3 g. of each compound to be evaluated

Suggested plant material—Use plants known to be highly sensitive to the compound to be tested. If the sensitivity of plants to the chemical is not known, select a number of plant species and determine useful ones on the basis of their sensitivity to direct application of the chemical to be tested.

Preparation and selection of plant material—Sort the seeds, eliminate any that are defective.

Procedure—The amount of the chemical used in these tests will depend on the specific compound tested. A mixture of 0.25 mg. of a chemical with 500 g. of a clay loam soil is equivalent to 0.5 kg. of the chemical applied to 4,047 sq. m. of clay loam soil and mixed to a depth of 18 cm.

Insoluble compounds, or compounds that are dry and of small particle size, can be dry-mixed with the soil before placing the chemical-soil mixtures in the containers. Use a total of 500 g. of soil per container.

To estimate the amount of moisture to be maintained in each container of soil, punch 10–12 holes in the bottom of one can with a 10-penny nail. Place 500 g. of soil in the can and record the total weight of can plus soil. Immerse the can in water to a depth of 3 cm. and maintain the water at this level until the sur-

face of the soil becomes moist. Remove the can containing the soil and allow it to drain 10–15 minutes. Record the weight of the can, water, and soil as the weight indicating the amount of moisture to be maintained. In conducting the experiment, add enough water each day or two to maintain this weight. Subtract the weight of the can plus soil from the weight of the can, soil, and moisture to obtain the initial weight of water to be added. To distribute the chemical throughout the soil in each container, dissolve the required amount of soluble compound in the above-determined weight of water and pour this evenly over the surface of the soil.

An alternative method that provides even distribution of the chemical throughout any soil can be used with any compound that is soluble in water or suitable organic solvents (acetone, alcohol, etc.). Dissolve a measured amount of the test compound in 6 ml. of a suitable solvent. Addition of a wetting agent, such as 0.4 ml. of Tween 20, may be advantageous. Add 20 g. of fine, clean quartz sand to this solution and stir to make a slurry. Dry this mixture at room temperature. Add the chemically coated sand to enough soil to make a total of 500 g. and mix it thoroughly before placing the treated soil in the container.

After the seeds have been planted, bring the soil samples to the correct moisture level. The number of seeds to be planted in each can will depend on the size of the plant.

Grow the test plants for 30 days. Then measure the height of the plants in the treated and untreated containers, cut off the plants at the soil level, and determine the fresh weight of the tops. Return the tops to the cans in which they were originally grown and, after a 30-day drying period, pulverize and replace the soil, putting the now dry tops beneath the soil. Replant the soil and bring it to the correct moisture level. Six crops a year can be grown in this manner to determine the residual effects of the compounds.

A variation of this test is as follows: Treat the soil with the test chemical as above but do not plant all of the containers at once. Store some of the containers for $\frac{1}{2}$, 1, and 2 months under different conditions. Conditions that can be altered easily are soil temperature and moisture content. Combinations of these conditions can be used, but it is strongly suggested that the conditions selected closely approximate the normal variation found in the field.

Method of taking results—The weight and height measurements taken after each succes-

sive growth period are compared. Comparative studies of residual effects can be made following use of one chemical and many soil types, or several chemicals in a single or a few soil types.

Suggested standard for comparison—No standard treatment suggested.

References

- Loustalot, A. J., and Ferrer, R. Studies on the Persistence and Movement of Sodium Trichloroacetate in the Soil. *Agronomy Jour.* 42: 323–327. 1950.
Robbins, W. W., Crafts, A. S., and Raynor, R. N. *Weed Control*. Ed. 1, 503 pp. McGraw, Hill Book Co., New York. 1942.

SOME PHYSICAL METHODS

INTRODUCTION

Physical methods, such as gas chromatography and measurement of radioactivity, are used widely for detection and identification of exogenous regulating substances in plants. Some chemical methods are also useful in this way. We are, however, not attempting to describe these physical and chemical methods comprehensively here. On the other hand, because of their importance in the study of growth regulators, radioactive isotopes must be at least briefly dealt with.

The use of radioactive tracer methods is based on the generally valid assumption that the active isotope behaves biologically in the tagged molecule exactly as does the inactive counterpart in a similar untagged molecule. An important characteristic of radioactive tracer methods is their extreme sensitivity when used to detect and measure tagged compounds. For instance, satisfactory quantitative measurement of C^{14} can be obtained on 2×10^{-11} g. of this substance.

Isotopes emitting alpha particles and beta and gamma rays have proved very useful in detecting tagged growth-regulating substances in entire plants and to some extent in thin sections of plant parts by means of radioautographs. As a general rule, adequate images are obtained on

film when as little as 4×10^{-15} g. of C^{14} per cm.² is exposed to the film for about one month.

In using radioisotopes, it must be kept in mind that degradation of at least some regulating substances, such as naphthaleneacetic acid, may occur on the surface of the plant even before the regulator is absorbed. Furthermore, absorbed regulating substances are sometimes altered within the plant in many ways. Because of these changes which result in degradation products or metabolites, it is desirable, whenever possible, to identify the molecule with which the tag is associated once the compound enters the plant.

Comprehensive information concerning isotope techniques and other physical and chemical methods of studying regulating substances can be found in references listed at the end of this section. Since radioactivity is used so widely to study regulating substances, some representative isotope techniques are described here.

Some radioactive materials are dangerous. Experiments with radioactive substances should not be undertaken without a thorough knowledge of the hazards involved.

RADIOAUTOGRAPHS OF ENTIRE PLANTS

RADIOAUTOGRAPH METHOD

Based on research by Shogo Yamaguchi and A. S. Crafts; variation by Edgar Inselberg.

Description of method—Compounds labeled with beta ray emitters are applied to leaves or to the culture medium where they are absorbed by the roots. The plant is later freeze-killed and freeze-dried under vacuum. X-ray film is then

exposed to the plant for the required period. The image obtained on the film is registered with the dried plant and the exact position of the tagged compounds recorded.

Apparatus, Chemicals, and Other Materials

Test tubes, 10 × 75 mm.

Micropipette, 10-lambda

Lanolin stiffened with granular starch or water

Small wire loop
 Facial tissues
 Dry ice
 Aluminum foil
 Masking tape
 Hardware cloth tray large enough to support plant involved
 Cardboard box, 15–20 cm. deep, large enough to contain the hardware cloth tray
 Vacuum tank
 Freezing compartment maintained at approximately -15°C .
 Vacuum pump
 Vacuum hose
 Vapor trap with freezing mixture of dry ice and alcohol
 Humidity chamber
 Glossy white paper
 Rubber cement
 Blotters
 Sheets of plywood
 Plant press
 X-ray film, 25×30 or 36×43 cm.
 Sponge rubber, 0.6 cm. thick
 Elastic web belts
 Light-tight box or light-proof wrapping material
 Dark room
 X-ray film development supplies and equipment
 Treatment solution containing approximately 0.05–1.0 microcurie of the labeled substance per 10 microliters of 50% ethyl alcohol and 0.1% Tween 20 in water

Suggested plant material—Any kind of plant can be used and a radioautograph can be made of any part of a plant, provided the part is of suitable size.

Preparation and selection of plant material—Select plants to be treated that are growing vigorously since rapid growth is associated with active transport. For leaf treatments, select leaves that are three-fourths or fully expanded.

Procedure—With a wire loop apply lanolin-starch paste to make a ring of the lanolin mixture, about 6 mm. in inside diameter, near the base of the leaf. (See also, "Application of a measured amount of regulating chemical to a known area of leaf surface," p. 98.) With the micropipette apply a measured amount of the labeled compound in solution covering completely the area within the lanolin ring. For full development of the distribution pattern, allow 2 days for a fast-growing plant and 4–10 days for others.

After this period, carefully wipe off the lanolin paste with tissue, making certain that the

tagged compound does not contaminate any parts of the plant. To guard further against contamination of other areas, cover the treated area on the leaf with masking tape or remove the entire treated area. (See "Separation of leaf areas treated with a regulating chemical from the remaining untreated portion of the leaf," p. 123.) Place the plant on a hardware cloth tray.

Place the hardware cloth tray into the vacuum tank which is then placed in a freezer compartment at -10° or -15°C . Connect the tank with a vacuum line, and evacuate it. Protect the vacuum pump from moisture by placing a vapor trap with a dry ice-alcohol mixture in the line leading from the tank to the pump.

Allow 4–10 days' drying time with pressures of 0.02–2.0 mm. of mercury. After the plant is dried, place it in a humidity chamber until it becomes flexible. Mount the plant with rubber cement that has not thickened on glossy white paper and press overnight between blotters in a press board. Place the mounted plant against an X-ray film. Sandwich the film and plant between sheets of sponge rubber and between 2 pieces of plywood and wrap the entire assembly with aluminum foil. If many plants are involved, repeat the procedure, forming a stack of sandwiches. Fasten an elastic belt around the stack and store it in a light-tight box for 2 wk. At the end of the storage period, develop the film using standard methods but reduce the time of development by one-half to three-fourths to obtain relatively light backgrounds.

Method of taking results—Place the radioautograph against glossy white paper and record the position of the darkened image that shows distribution of the labeled compound. Determine the exact location of the labeled compound by comparing the darkened image with the pressed and dried plant material.

Suggested standard for comparison—No standard treatment suggested.

Variation used by Inselberg—Another method which might be of interest when high-energy beta, low-energy gamma, or X-ray radiation is involved provides for using the film without removing it from its original container, thus eliminating the need for light-proof equipment.

References

- Crafts, A. S., and Yamaguchi, Shogo. Gross Autoradiography of Solute Translocation and Distribution in Plants. *Med. and Biol. Illus.* 10: 103–109. 1960.
 Inselberg, Edgar. Simplified Procedures for Radioassay and Autoradiography of Plant Materials. *Agron. Jour.* 51: 301–306. 1959.
 Yamaguchi, Shogo, and Crafts, A. S. Autoradiographic Method for Studying Absorption and Translocation of Herbicides Using C^{14} -Labeled Compounds. *Hilgardia* 28: 161–191. 1958.

TRANSLOCATION OF RADIOACTIVELY TAGGED COMPOUNDS

GROUND TISSUE METHOD

Based on research by J. W. Mitchell and P. J. Linder.

Description of method—Translocation of radioactively tagged molecules is measured by placing a tagged compound on one part of a plant and measuring radioactivity that results from the movement of the compound, metabolites, or degradation products of it to other parts of the plant. Radioactivity in dried, ground, plant tissue is measured directly. Since self-absorption is involved, the method is only reliable for comparison of radioactivity in comparable tissues ground to approximately the same particle size.

Apparatus, Chemicals, and Other Materials

Conventional Geiger tube
Q-gas counter (or counter of similar sensitivity if carbon-tagged compounds are used)
Wiley mill
Scaler
Lead tube for shield
Remote control applicator (fig. 34)
Brass rings (inside diameter 25 mm., outside diameter 31 mm., 3 mm. high)
Cellophane tape, 4 cm. wide
Analytical balance
0.5–1.0 mg. of 2,4-dichloro-5-iodophenoxyacetic acid tagged with I^{131} , one of its salts, or 2,4-dichlorophenoxyacetic acid tagged with C^{14} for standard treatment
Lanolin
Tween 20

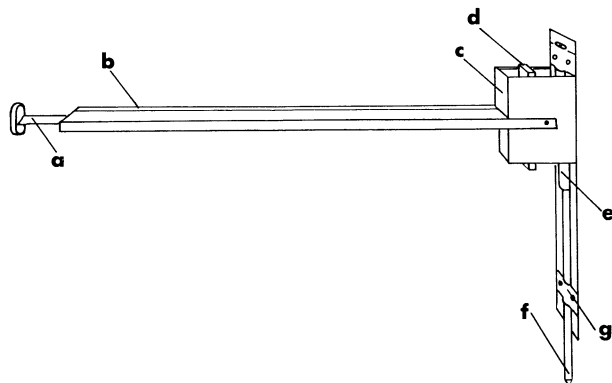


FIGURE 34.—Diagram of remote-control applicator used to apply small volumes of solutions containing radioactive regulating chemicals to leaves and stems of plants.

Suggested plant material—Any kind of terrestrial plant.

Preparation and selection of plant material—The following method is designed for use in studying translocation from leaves of plants such as bean (*Phaseolus vulgaris* L.), tomato (*Lycopersicon esculentum* Mill.), or other plants commonly grown in a greenhouse, but it can be modified for application to any part of a terrestrial plant. Plant seeds in soil contained in clay pots and allow several plants to grow in each pot until the primary leaves or the first secondary leaves have become photosynthetically active and capable of supplying photosynthate to other parts of the plants. Thin the plants to one in each pot, retaining only plants of uniform size.

Procedure—Make a remote control applicator as follows: Support an aluminum rod (fig. 34, a) approximately 65 cm. long and 5 mm. in diameter with two 13-mm. aluminum angles (b). Thread one end of the rod and screw it through a plate into a metal box (c) so it rests against a movable plate (d). Turn the rod to press the plate against the piece of soft rubber tubing (e), which is held in place by clamps. Close the upper end of the tubing with a glass plug. Insert a 0.1-ml. pipette (f) in the lower end of the tubing and fasten the pipette with a clamp (g). Turn the rod and expel the air from the pipette; then replace it with a solution containing the radioactive growth regulator. Force a measured portion of the solution (0.005, 0.01, or 0.02 ml.) out of the pipette by turning the rod, and apply the drop by touching the tip of the pipette to the surface of a plant. When necessary, use the tip of the pipette to spread the liquid evenly over the plant surface.

Prepare an aqueous mixture which contains 0.1% of Tween 20 (or other suitable cosolvent) and 10 μ g. of the radioactive plant regulator per 0.01 ml. This can be easily accomplished, using the proper precautions, by weighing the required number of mg. of the radioactive substance into a vial and adding the required amount of distilled water containing the cosolvent. With a short piece of glass rod 3–5 mm. in diameter, crush all crystals of the growth regulator and stir until the very fine pieces dissolve.

Use a short piece of rubber tubing (6–7 mm. inside diameter) and stamp a ring of lanolin on the upper surface of the leaf enclosing an area in the desired position. (See p. 98.) With the remote control applicator, place 0.01 ml. of the

mixture within the lanolin ring. If the entire upper surface of the leaf is to be covered with the mixture, use the method described on p. 12. Replicate treatment with any one tagged compound at least five times, using an equal number of comparable plants.

After application of the radioactive regulator, place the plants in a well-illuminated greenhouse, or, if necessary, illuminate them with fluorescent tubes so they receive at least 700 foot-candles of light at the leaf surface. After the desired length of time (from a few hours to several days) remove the treated leaves very carefully and place them in a paper bag to be discarded. Wash the remainder of the plants free of soil, divide them into roots, stems, etc. Dry each part in a well-ventilated oven and grind each sample to 40 mesh, exerting care that one sample does not become contaminated by another.

The size and density of the tissue particles that make up a sample of ground plant material control, to some extent, the amount of self-absorption of radioactivity that occurs within the sample. It is difficult to grind to comparable particle size tissues from plants grown under widely different environmental conditions or tissues from different parts of plants grown under comparable conditions. The amount of fiber and secondary thickening that develops in stems and leaves depends to some extent upon the environmental conditions to which the plants are subjected. Plants grown in relatively high light intensity often contain proportionally more fiber than do those grown in shade, and the former are, therefore, more difficult to grind to a small particle size.

More mature parts of stems generally contain proportionally more fiber than do relatively immature parts of stems, and samples of the former are, therefore, difficult to grind into particles of relatively small size. Care must be exercised in the utilization of this method so self-absorption, due to differences in size and density of particles, does not introduce significant errors.

Method of taking results—To determine the radioactivity in a sample, place a brass ring on the tacky surface of the cellophane and trim off the cellophane flush with the outside of the ring; then determine the weight of the ring and cellophane. Thoroughly mix the powdered sample and place a small portion of it on the tacky surface of the cellophane. By gently tapping the ring, roll the powdered tissue back and forth until the entire surface is evenly covered. Shake the excess of tissue into its original container and weigh the ring, cellophane, and adhering tissue to determine the amount of tissue used to measure the radioactivity. Place the ring in the holder of the counter and determine the number of counts per minute that emanate from the sample. Calculate net counts per milligram per minute by subtracting background counts.

Suggested standard for comparison—2,4-Dichloro-5-iodophenoxyacetic acid tagged with I^{131} , one of its salts, or 2,4-dichlorophenoxyacetic acid tagged with C^{14} .

Reference

Mitchell, J. W., and Linder, P. J. Some Methods Used in Tracing Radioactive Growth-Regulating Substances in Plants. *Bot. Gaz.* 112: 126-129. 1950.

VOLATILITY ⁵ OF PLANT REGULATORS

DISPOSABLE CONTAINER METHOD

Based on research by P. C. Marth and J. W. Mitchell.

Description of method—This method is useful for detecting volatility of plant-regulating compounds that induce easily detectable growth responses. It can be used to detect or measure evaporation of regulating chemicals from surfaces of plants previously treated with these

compounds or from surfaces of other materials to which these substances have been applied.

Apparatus, Chemicals, and Other Materials

Clay or paper pots, 8 cm., or clean discarded tin cans, about 450-g. capacity

Analytical balance

Greenhouse space maintained at 24°-29° C. for growing test plants

Table or bench space in darkened room maintained at 21°-32° C. for conducting tests

Several 1- and 5-ml. pipettes

Vials, 20-30 ml. with tightly fitted stoppers or screwcaps

⁵ Carelessness in the use or disposal of highly volatile regulating chemicals can result in contamination of an entire laboratory or greenhouse, thus causing loss of valuable plants and space.

Filter paper No. 1 or other absorbent paper disks, 7–9 cm. in diameter

Cellophane bags, 10 cm. square and 41 cm. tall

Adhesive cloth, plastic tape, paper clips, or electric iron for sealing cellophane bags

95% ethanol or acetone for solvent

0.1–1 g. of each pure compound to be tested
1–2 g. of the butyl or isopropyl ester of 2,4-dichlorophenoxyacetic acid for standard treatment

Suggested plant material—Determine a suitable test plant by placing a small amount of the chemical to be tested on several kinds of young plants; suggested are snap bean (*Phaseolus vulgaris* L.), buckwheat (*Fagopyrum esculentum* Moench), cucumber (*Cucumis sativus* L.), and tomato (*Lycopersicon esculentum* Mill.).

Preparation and selection of plant material—Plant several seeds of bean, tomato, or other test plant in soil contained in disposable pots or containers. Employ optimum greenhouse conditions of light, temperature, and moisture to produce rapidly growing seedlings for the tests. Select bean plants that have primary leaves measuring 3 cm. across, and with trifoliate leaves still folded in the terminal buds (about 5–7 days old); or tomato plants 6.4–7.6 cm. tall (about 25 days old). Thin the plants, leaving a single plant, all of uniform size, in each pot.

Procedure—Prepare an alcohol or acetone solution of the pure chemical by placing a weighed amount of the chemical in a vial and adding sufficient 95% ethanol or acetone to give the desired concentration. For example, weigh 5 mg. of the chemical into a vial and add 25 ml. of solvent to obtain a solution containing 0.2 mg. of the compound per ml.

Impregnate a filter paper or other suitable paper disk with 1 ml. of the alcohol mixture, and allow the solvent to evaporate completely (5–30 minutes). Use 3 or more replications for each compound tested.

Place a pot containing the selected test plant upright in the cellophane bag. Fasten the impregnated filter paper with a small piece of cellophane tape inside the bag, about 5 cm. from the top. Do not allow the impregnated filter paper to come in contact with the plant during the test. Close the open ends of the cellophane bags and fold them downward at a distance of about 2.5 cm.; then seal with a hot iron.

Expose the plants to the vapor from the test chemical for the desired length of time (24–72

hr.) and at room temperature; then remove, and allow them to remain in a greenhouse for later observation. Dispose of the filter paper containing the chemical, bags, etc., so as to avoid contamination of the room or greenhouse.

Method of taking results—The response of the plant (leaf and stem curvature) is due to two separate factors; first, the ability of the chemical to induce the response when in direct contact with the plant; and second, the rate that the chemical evaporates from the paper and comes in contact with the plant. Since this method is based both on the biological effectiveness of the compound and its ability to evaporate, the method cannot be used to measure either of these factors separately.

Immediately before removal of the plants from the bags, record the degree of curvature, comparing the plants with a diagram or photograph illustrating typical plants that have responded slightly, moderately, or to a marked degree. For convenience, assign these categories numerical values such as 0, 1, 2, 3, and 4. Carefully dispose of any remaining contaminated materials.

Suggested standard for comparison—Impregnate a filter paper disk with 0.2 mg. of butyl ester of 2,4-dichlorophenoxyacetic acid and expose the plants exposed to the vapors for 48 hr. at 27°–32° C.

Reference

Marth, P. C., and Mitchell, J. W. Comparative Volatility of Various Forms of 2,4-D. Bot. Gaz. 110: 632–636. 1949.

GERMINATING SEED METHOD

Based on research by W. P. Anderson.

Description of method—Relative volatility of regulating compounds is determined by exposing germinating seeds to vapors of these chemicals in closed containers.

Apparatus, Chemicals, and Other Materials

Petri dishes, about 15 cm. in diameter × 2 cm. deep and 5 cm. in diameter × 2 cm. deep

Filter paper, Whatman No. 1, to fit the petri dishes

Forceps

Wide-mouth moisture- and air-tight containers, 0.5–1 liter

Constant-temperature box or room controlled at 21°–24° C.

Analytical balance

250–500 mg. of each compound to be evaluated

Highly volatile solvent such as acetone, ether, or ethanol

Compounds known to have a relatively high degree of vapor activity, such as isopropyl ester of 2,4-dichlorophenoxyacetic acid and isopropyl N-(3-chlorophenyl) carbamate for standard treatment

Suggested plant material—Cucumber (*Cucumis sativus* L.), buckwheat (*Fagopyrum esculentum* Moench), corn (*Zea mays* L.), or other seeds.

Preparation and selection of plant material—Germinate seeds in the large petri dish containing moistened filter paper at a temperature of about 21°–24° C. When the seed coat has cracked and the tip of the radicle is visible through this opening, select 5 seeds at a uniform stage of germination. Place these on moistened filter paper in the small petri dish and then place the dish in the air-tight container. In the case of cucumber and buckwheat seeds, this stage is reached 17 and 20 hr., respectively, after the seeds are placed in moist petri dishes.

Procedure—Impregnate a disk of filter paper (5 cm. in diameter) with 50 mg. of the chemical to be tested, dissolved in highly volatile solvent. After evaporating the solvent, place the filter paper in the air-tight container in such a manner as to avoid direct contact with the petri dish containing the germinating seeds. Close the container tightly and place it in the constant-temperature box along with others for the same test. The period of exposure may vary from a few to about 48 hr., depending upon the type of response to be measured and the relative rates of evaporation of the chemicals.

At the end of the designated period, remove the petri dishes from the containers, cover the dishes, and allow the seedlings to grow for several more days; observe variations in growth caused by the treatment.

Method of taking results—Make visual comparisons of the response of the seedlings, noting increase or decrease in length of the root hair zone, the relative number of hairs, increase or decrease of branching along the taproot, the relative lengths of the taproot and branch

roots; abnormalities occurring in the growth of the hypocotyl.

Suggested standard for comparison—Compounds known to have a relatively high degree of vapor activity, such as isopropyl ester of 2,4-dichlorophenoxyacetic acid and isopropyl N-(3-chlorophenyl) carbamate.

Reference

Unpublished method submitted by W. P. Anderson, Amchem Products, Inc., Ambler, Pa.

ACTIVATED CHARCOAL METHOD

Based on research by P. J. Linder.

Description of method—Measured amounts of the radioactive test chemical are applied to filter paper pads suspended in a closed chamber containing activated charcoal. Volatility of the compound is determined by subsequently measuring the radiation emanating from the test chemical adsorbed on the activated charcoal.

Apparatus, Chemicals, and Other Materials

Thin copper wire
Fruit jar (approximately 1 liter) or similar glass chamber that can be tightly closed
Sealing wax
Filter paper
Brass ring to fit the radioactivity chamber to be used
Cellophane tape, 3 cm. wide
Activated charcoal
Aluminum foil
Conventional equipment for detection of radioactivity
0.25 g. of compound (radioactively tagged) to be evaluated
250 micrograms of C¹⁴-tagged methyl 2,4-dichlorophenoxyacetate for standard treatment
Alcohol

Procedure—Attach 4 wires to the inner surface of the fruit jar lid, using sealing wax (fig. 35, *a*). Cut uniform disks of filter paper (*c*) 1–2 cm. in diameter. Pierce each of these near the edge with the end of one wire (*b*), bending the wire so that each disk will remain suspended separately within the jar. Dissolve 0.5 mg. of each tagged chemical to be tested per 0.05 ml. of solvent (alcohol). Place 0.05 ml. of the alcohol solution of the regulator on each paper disk. Prepare a brass ring (*f*) covered

with cellophane tape. (See "Ground tissue method," p. 89.) Cover the surface of the tape with activated charcoal. Place the brass ring in the bottom of the jar and support a piece of aluminum foil (*d*) above the brass ring to protect the charcoal from any radioactivity that might accidentally fall from the impregnated paper disks. Close the chamber (*e*) tightly and allow it to stand for a period from 30 minutes to overnight.

Method of taking results—Remove the brass ring and record any radioactivity present using the conventional equipment for detection of radioactivity.

The possibility exists that the growth regulator being tested may be contaminated with a volatile substance, the regulator itself being nonvolatile. It is, therefore, suggested that the radioactive compound adsorbed to the activated charcoal be dissolved and chromatographed along with an aliquot of the original sample. Compare Rf's of chromatographed samples by means of radioautographs to determine whether one or more compounds are involved.

Suggested standard for comparison— C^{14} carboxyl-tagged methyl ester of 2,4-dichlorophenoxyacetic acid.

Reference

Method submitted by P. J. Linder, Agricultural Research Service, U.S. Department of Agriculture.

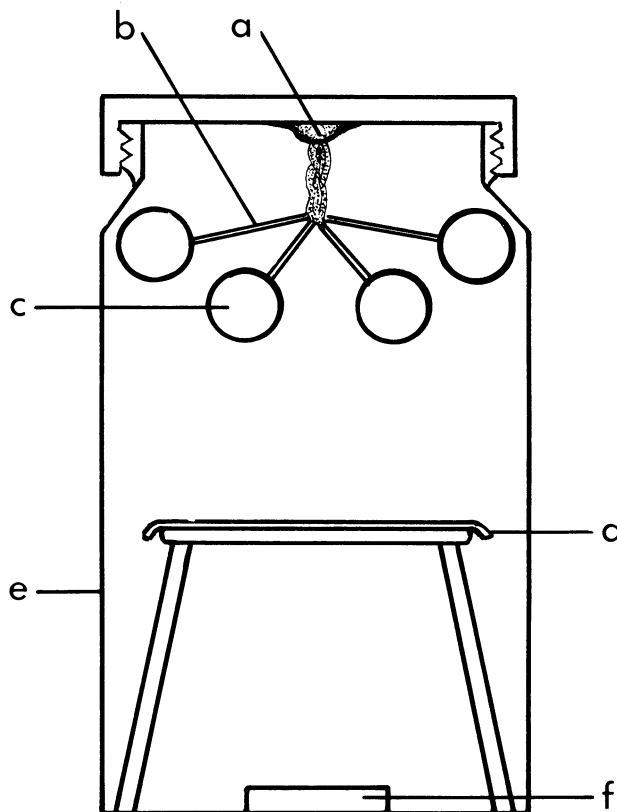


FIGURE 35.—Closed system used to measure volatility of radioactively tagged regulating substances.

REFERENCES TO OTHER PHYSICAL METHODS

The publications listed below are recommended for those wishing to gain a more thorough understanding of how radioisotopes can aid in the study of growth-regulating substances. For extraction of these, see p. 118.

- Aronoff, Sam. *Techniques of Radiobiochemistry*. 145 pp. Iowa State College Press, Ames. 1956.
- Biddulph, Orlin. *Translocation of Radioactive Nutrients in Plants*. Conf. Use of Isotopes in Plant and Animal Research, U.S. Atomic Energy Comm., TID-5098 (April 1953); Agr. Expt. Sta., Kans. State Col., Rpt. 4: 48-58. 1953.
- Boyd, G. A. *Autoradiography in Biology and Medicine*. 399 pp. Academic Press, Inc., New York. 1955.
- Calvin, M., Heidelberger, C., Reid, J. C., and others. *Isotopic Carbon*. 376 pp. John Wiley & Sons, New York. 1949.
- Comar, C. L. *Radioisotopes in Biology and Agriculture*. 481 pp. McGraw-Hill Book Co., New York. 1955.

- Hevesy, Georg von. *Radioactive Indicators*. 556 pp. Interscience Publishers, Inc., New York. 1948.
- Overman, R. T., and Clark, H. M. *Radioisotope Techniques*. 476 pp. McGraw-Hill Book Co., New York. 1960.
- Schweitzer, G. K., and Whitney, I. B. *Radioactive Tracer Techniques*. 241 pp. D. van Nostrand Co., Inc., New York. 1949.
- U.S. National Bureau of Standards. *Safe Handling of Radioactive Isotopes*. Natl. Bur. Standards Handb. 42, 30 pp. 1949.
- U.S. National Bureau of Standards. *Control and Removal of Radioactive Contamination in Laboratories*. Natl. Bur. Standards Handb. 48, 24 pp. 1951.
- Wolf, George. *Isotopes in Biology*. 173 pp. Academic Press, Inc., New York. 1964.
- Yagoda, H. J. *Radioactive Measurements with Nuclear Emulsions*. 356 pp. John Wiley and Sons, New York. 1949.

CHROMATOGRAPHY

Chromatography is an essential part of many methods involved in the study of hormones and regulating chemicals. All of the major types of chromatography, including paper, column, gas, and thin-layer techniques, have played an important role in our gaining an understanding of hormones and regulating substances. For example, with chromatography it is possible to determine the purity of a regulator before it is applied to plants and to purify and identify hormones obtained from plants. With chromatography it is possible to determine whether an externally applied substance is absorbed and translocated as such, or whether some of the applied substance is degraded, how it is degraded, and, finally, what happens to the products of degradation. Therefore, investigators in the field of hormones and regulating substances must have a thorough knowledge of the methods and techniques involved in chromatography and must be skilled in the application of these methods wherever they are needed.

Although chromatography is mentioned in many of the methods described in this book, it is not possible, nor necessary, to give chromatographic procedures in detail here. Excellent theoretical considerations and descriptions of chromatographic techniques can be found in the following and other texts. (See also p. 118 for hormone extraction methods.)

References

- Block, R. J. *A Manual of Paper Chromatography and Paper Electrophoresis*. 2nd ed., rev. and enlarged. 710 pp. Academic Press, Inc., New York. 1958.
- Block, R. J., Le Strange, Raymond, and Zweig, Gunter. *Paper Chromatography, A Laboratory Manual*. 195 pp. Academic Press, Inc., New York. 1952.
- Bobbitt, J. M. *Thin-Layer Chromatography*. 208 pp. Reinhold Pub. Corp., New York. 1963.
- Burchfield, H. P., and Storrs, Eleanor E. *Biochemical Applications of Gas Chromatography*. 680 pp. Academic Press, Inc., New York. 1962.
- Dal Nogare, Stephen. *Gas-Liquid Chromatography, Theory and Practice*. 450 pp. Interscience Publishers, Inc., New York. 1962.
- Hais, I. M., ed. *Paper Chromatography; A Comprehensive Treatise*. Ed. 3, 955 pp. Publishing House of the Czechoslovak Academy of Science, Prague. 1963.
- Heftmann, Erich, ed. *Chromatography*. 753 pp. Reinhold Pub. Corp., New York. 1961.
- Keulemans, A. I. M. *Gas Chromatography*. Ed. 2, 234 pp. Reinhold Pub. Corp., New York. 1959.
- Linskens, H. F. *Papierchromatographie in der Botanik*. 408 pp. Springer-Verlag, New York. 1959.
- Littlewood, A. B. *Gas Chromatography; Principles, Techniques and Applications*. 514 pp. Academic Press, Inc., New York. 1962.
- Pollard, F. H., and McOmie, J. F. W. *Chromatographic Methods of Inorganic Analysis*. [With special reference to paper chromatography.] 192 pp. Butterworths Scientific Publications, London. 1953.
- Randerath, Kurt. *Thin-Layer Chromatography*. 250 pp. Academic Press, Inc., New York. 1963. [Translated by D. D. Libman.]
- Stahl, Egon, ed. *Thin-Layer Chromatography, A Laboratory Handbook*. 553 pp. Academic Press, Inc., New York. 1965.

SUPPLEMENTARY TECHNIQUES

Some techniques developed by scientists are of general use in studying responses of plants to regulating chemicals. A number of these are described on the following pages. Some of the less complex ones may be of particular interest to those who are undertaking work with regulating chemicals for the first time. Other more

complicated techniques are described with the hope that they will be useful to the more advanced research worker. It is not possible to determine the originators of all of the techniques listed; therefore, credit for some of them is not given.

TREATING PLANTS

Absorption Cell for Introducing Compounds Into Limited Portions of Roots

Construct an absorption cell for applying radioactively tagged chemicals to limited regions of individual roots by using a glass tube 10 mm. in diameter with a 3-mm. sidearm. Draw the end of the larger tube out to make

it smaller (about the diameter of the sidearm), and bend it around so the narrow end faces the sidearm as shown in fig. 36. Connect this narrow end of the tube with the sidearm using pure gum amber rubber tubing 3 mm. in diameter. Burn a hole through both walls of the rubber tubing with a hot nichrome wire of

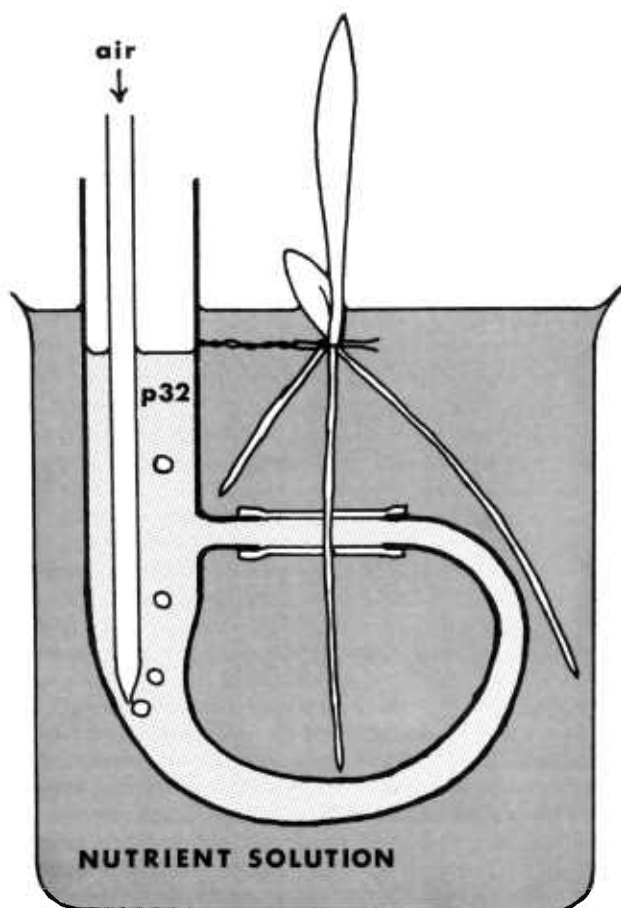


FIGURE 36.—Absorption cell for introducing compounds into limited portions of roots. (Photo—courtesy of Plant Physiology.)

somewhat smaller diameter than the roots. The size of the hole should be slightly smaller than the root so the root fits snugly. Connect the holes by a slit. Bend the rubber tubing slightly to open the slit and insert a rootlet of an intact plant. Seal the slit in the tubing and the junction between the tubing and the root with lanolin that has been warmed to make it almost liquid. Twist a piece of nichrome wire to make a support for the plant as illustrated. Place the solution of the chemical to be absorbed by the root in the cell and immerse the apparatus in tapwater or a nutrient solution as illustrated. Tap the apparatus gently to dislodge air bubbles trapped in the rubber tubing. Insert glass tubing in the main tube of the cell and bubble air through this tube to circulate and aerate the solution in the cell. Keep the level of the solution in the cell 1.3 cm. below the level of external nutrient solution so that, if leaks develop, they can be de-

tected by the rise in the level of the solution within the cell. Aerate the external nutrient solution. (See "Plastic culture vessel ("plasticon") for small plants," p. 107.) Change the nutrient solution frequently to keep to a minimum contamination due to exudation from the roots. Maintain the whole apparatus at 25° C.

It has been suggested that, with some modification, this method might be useful to indicate root uptake of regulators by determining the growth response of the plant after it absorbs the chemical. Absorption of the compound might also be detected by bioassay and chromatography of tissue extracts.

This technique was successfully used to introduce P^{32} , I^{131} , Rb^{86} , and S^{35} . It was not successful in introducing Ca^{45} . This method is described for workers who may wish to attempt its adaptation for use with radioactively tagged growth regulators.

Reference

Wiebe, H. H., and Kramer, P. J. Translocation of Radioactive Isotopes from Various Regions of Roots of Barley Seedlings. *Plant Physiol.* 29: 342-348. 1954.

Absorption Through a Leaf Flap

A flap absorption technique that has been used successfully is as follows: Place the leaf of an intact plant under water. Using a razor blade, cut parallel to and on both sides of the midrib of a leaf, such as that of a bean plant, for a distance of 2-3 cm. (fig. 37, a). Connect

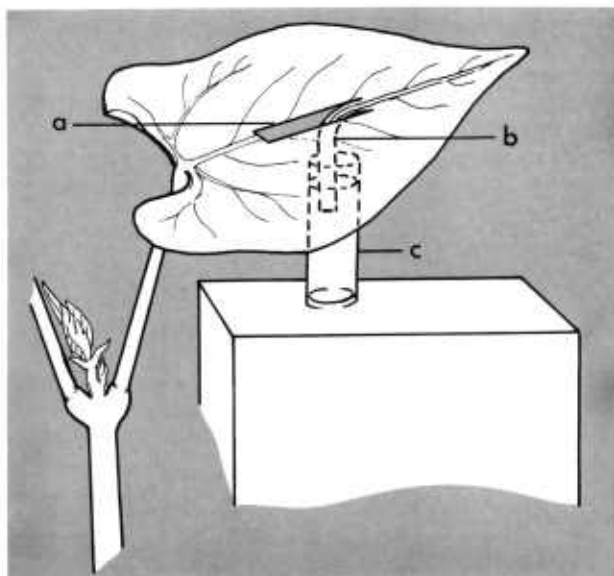


FIGURE 37.—Leaf-flap absorption technique.

the 2 cuts at the ends nearest the petiole attachment while the leaf is still immersed. Remove the leaf from the water and dip the flap (b) immediately into the test solution contained in a small vial (c) to prevent air from entering the xylem vessels. Allow the test solution to be absorbed for 1-24 hr. Remove the flap and immerse it in a vial containing water to prevent drying. Some absorbed test solutions will be translocated through the leaf and into the plant. One principal lateral vein of a bean leaflet has also been used for making the flap in a method similar to the one described above.

Reference

Biddulph, O. Diurnal Migration of Injected Radiophosphorus from Bean Leaves. *Amer. Jour. Bot.* 28: 348-352. 1941.

Agar Block Preparation and Use

Agar blocks are useful for applying exogenous substances and hormones to plants and plant segments used in bioassays and translocation experiments. When a moist agar block containing a test compound evenly dispersed throughout the agar is placed on a freshly cut plant surface, the compound in the agar block readily diffuses from the agar into the aqueous film between the agar and the plant surface. In most instances the compound then diffuses into the plant cells. Conversely, some endogenous substances, as well as exogenous substances that have been introduced into plants, will diffuse in a similar manner from freshly cut surfaces into an agar block. Thus, under suitable conditions, agar blocks represent a means of supplying substances to and collecting some substances from plants.

To make agar blocks, prepare 1% agar by mixing together and bringing to a boil 0.1 g. of the agar (ionagar) and 10 ml. of water. Pipette the hot agar mixture into a glass tube (15-30 cm. long, 5 mm. inside diameter). Hold a finger over the lower end of the tube to keep the agar in place. After the tube is full, place another finger over the upper end of the tube and hold the entire tube under a stream of cold running water. When the agar has solidified (1-2 minutes), tap the tube endwise gently on a hard surface to loosen the agar from the inside wall. Force the agar column from the tube with a solid glass rod. Repeat this process until sufficient molded agar has been obtained. Slice each column of agar into segments of appropriate length (2 mm.) with the common cutter and place them in a petri

dish lined with moist filter paper. Store the segments at 5° C. until used. (See p. 101.)

A somewhat more precise method has been suggested by McCready. Prepare a 2% agar solution and draw it into a number of lengths of precision glass tubing (3.0 mm. diameter). After the agar has solidified, insert a plug of nonabsorbent cotton into one end of each tube. Extrude the columns of agar from the tubes by pushing the cotton. Cut the columns into blocks of appropriate length. Use the common cutter with the desired number of separator plates and a corresponding number of razor blades, the width of each plate adjusted to give the desired length of agar block. After cutting the columns, use a comb with teeth properly spaced to displace alternate blocks from between the blades. Move the comb slightly and remove the remaining blocks so that 2 rows of blocks are conveniently spaced for handling with forceps. If the agar contains a growth substance, use a separate set of cutter blades and a different comb and forceps when preparing and handling receiver blocks of plain agar.

A variation in the preparation of agar blocks and the method of supporting the blocks and segments was used by McCready. Prepare a 2% agar solution and draw this solution into a number of lengths of precision glass tubing (3.0 mm. diameter). Allow the agar to solidify. Insert a plug of nonabsorbent cotton into one end of each tube. Push the cotton through the tube extruding the column of agar gel. Cut the gel column into blocks of the desired length using the common cutter with the required number of blades spaced 2-3 mm. apart. Use a comb with teeth properly spaced to move alternate blocks from the cutter onto a glass plate. Move the cutter slightly and expel the remaining blocks so that 2 rows of blocks are conveniently spaced for picking up with forceps. If the agar contains a growth-regulating substance, do not use the same cutter, combs, and forceps as those used with receiver blocks of plain agar. Place the donor block on the apical end of the plant segment and the receiver block at the basal end of the segment. Lay each segment, with its blocks retained in position by surface tension, horizontally across a 3-mm. gap between 2 glass strips cemented to a glass plate. Prepare as many replications of this treatment as required. Repeat the procedure, this time applying the donor block to the basal end and the receiver block to the apical end of the segment, arranging the segments across the 3-mm. gap between additional plates as described above. Use the replications equal to the number in

the first treatment. Place the plates with the segments and agar blocks in a moist atmosphere in a dark chamber at a controlled temperature of 25° C.

It is sometimes desirable to make blocks that will accommodate more than one plant segment. Leopold, Lam, and de la Fuente have suggested methods for doing this. To make equipment for this purpose, drill a hole 20 mm. in diameter in a brass or aluminum plate 2 mm. thick. Place this mold on a microscope slide that is cooled from below with ice. Pipette into the mold about 1 ml. of hot 1.5% agar solution. After the agar has solidified, remove the metal plate leaving the agar block on the slide. Place 10–20 segments of the desired plant material vertically on this agar block. Prepare a similar agar block that contains 10^{-5} M C^{14} -tagged indole-3-acetic acid or other radioactive compound to be tested. Carefully slide this block onto the standing plant segments so that the block is in contact with the upper end of each segment. Place the microscope slide and segments in a petri dish or plastic box lined with moistened filter paper and cover the dish or box. After the desired time interval, measure the amount of compound in the lower agar block using the method described in the "Agar block polar transport method," p. 8.)

It is sometimes convenient to substitute a disk of moist filter paper for the receiver block or to dry the agar receiver block and then measure directly the radioactivity in it, correcting for self-absorption. When direct scintillation counting of the receiver block is de-

sired, make blocks of an aqueous paste of Cab-o-Sil. To avoid the quenching effect of the agar in the scintillation solution, extract the blocks with ethanol and determine the radioactivity in the extract.

Diffusible endogenous hormones can also be collected from plant segments or plant parts by placing their cut surfaces against agar disks.

References

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 Leopold, A. C., and Lam, S. L. The Auxin Transport Gradient. *Physiol. Plant.* 15: 631–638. 1962.
 McCready, C. C. Movement of Growth Regulators in Plants. I. Polar Transport of 2,4-Dichlorophenoxyacetic Acid in Segments From the Petioles of *Phaseolus vulgaris*. *The New Phytol.* 62: 3–18. 1963.
 Zaerr, J. B. Submitted the principal polar transport method described. (See "Agar block polar transport method," p. 8.)

Application of Aqueous Mixtures Quantitatively to Stems

Apply two narrow lanolin bands about 1–2 cm. apart and extending around the stem of the selected plant. Allow 0.01 ml. of the aqueous mixture containing the regulating chemical to flow from a 0.1-ml. pipette so the measured drop clings to the tip of the pipette. Hold the stem in a horizontal position and transfer the drop from the pipette to the stem surface between the lanolin bands (fig. 38). Revolve the plant and spread the mixture over the

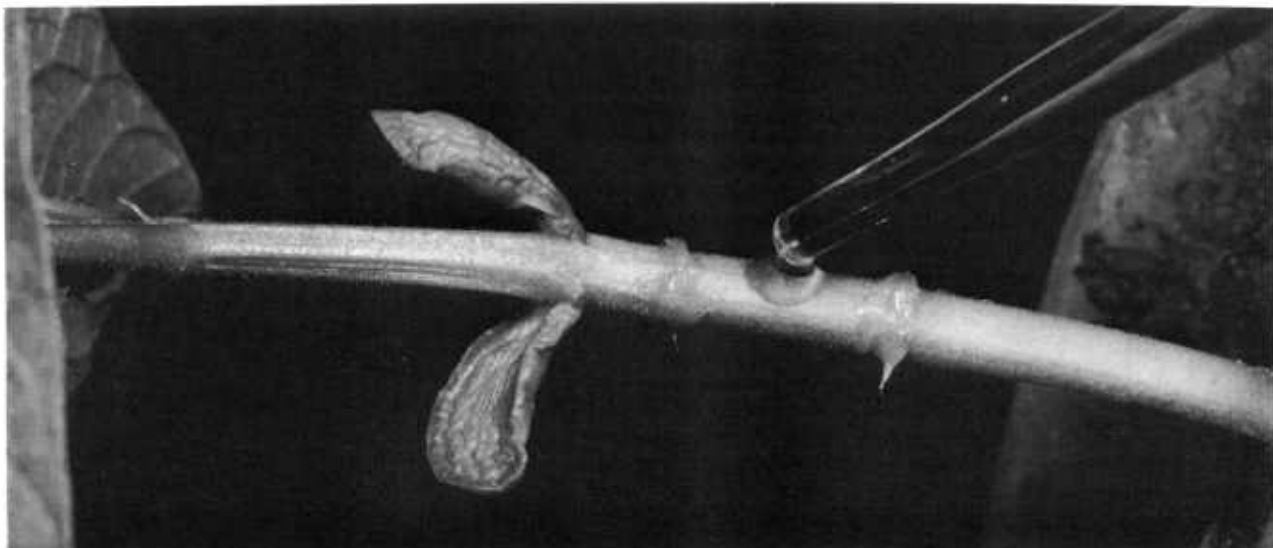


FIGURE 38.—Applying an aqueous mixture of a growth regulator quantitatively to a stem.

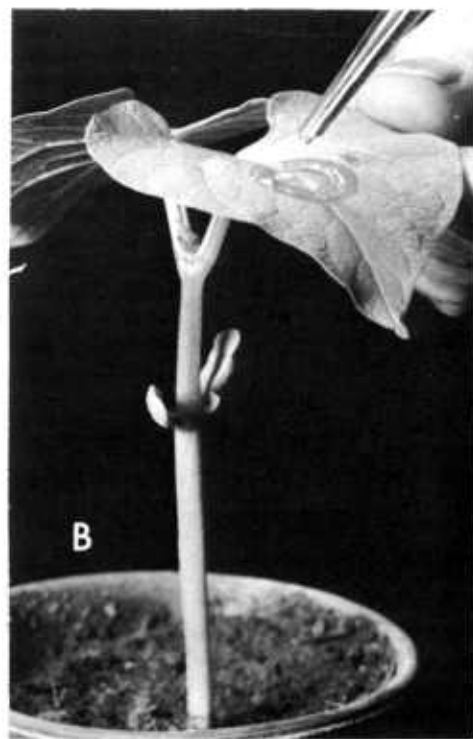
surface between the lanolin bands with the tip of the pipette. Keep the stem in a horizontal position and revolve the plant at frequent intervals so that the liquid remains evenly spread over the surface while it dries. Return the plant to an upright position and record growth effects that develop.

Application of a Measured Amount of Regulating Chemical to a Known Area of Leaf Surface

It is sometimes necessary to apply measured amounts of a regulating chemical to a limited area on a leaf, especially when radioactively tagged regulators are used. Slip a short piece of rubber tubing of appropriate diameter over the end of a cork borer of suitable size, leaving about 1 cm. of tubing extending beyond the end of the cork borer. Calculate the inside cross-sectional area of the rubber tube. Spread lanolin over the surface of a glass plate to a depth of about 1–2 mm. and press the end of the rubber tubing in the lanolin layer; then gently stamp a ring of the lanolin on the leaf surface (fig. 39, A). Fill a 0.1-ml. pipette (graduated in 0.01-ml. portions) with the aqueous mixture of the regulating chemical to be used, touch the tip of the pipette against a piece of glass or other hard surface to remove excess mixture, and then allow 0.01 ml. of the mixture to drain from the pipette so that the measured volume remains suspended at the end of the pipette. Touch the end of the pipette to the area enclosed by the lanolin ring and move the tip gently over the area until the measured volume of liquid is spread evenly over the enclosed surface (fig. 39, B). Aqueous mixtures of regulating chemicals spread over leaf surfaces most readily when a surfactant such as Tween 20 or Triton X 100 is present in the mixture at a concentration of 0.05 or 0.1%.

Application of Regulators as a Part of a Coating on Seeds

When it is desirable to apply regulating chemicals to seeds without the use of water, which generally initiates germination, the compounds can be mixed with various kinds of nonaqueous carriers and the mixture applied to the seeds as a coating. A typical seed-coating procedure, which can be varied readily, is as follows: Place the seeds in a rotating pan such as that used by pill and candy manu-



PN-306, A and B

FIGURE 39.—A, Marking a known area of a leaf with lanolin; B, applying a measured volume of solution containing a known amount of growth regulator to this area.

facturers.⁶ Wet the seeds with methyl cellulose, an adhesive material, and then dust the seeds with a mixture of 65% feldspar and 35% fly-ash (ash obtained from the burning of powdered coal) containing the required amount of growth regulator. Repeat the wetting and dusting procedure until the desired thickness of coating has been obtained. Use a coarse feldspar which passes through a 40-mesh screen to build up most of the coating. For a technique of coating individual particles of this coarse feldspar with the regulating chemical, see "Easy method of impregnating dust carriers with regulating chemicals," p. 100.

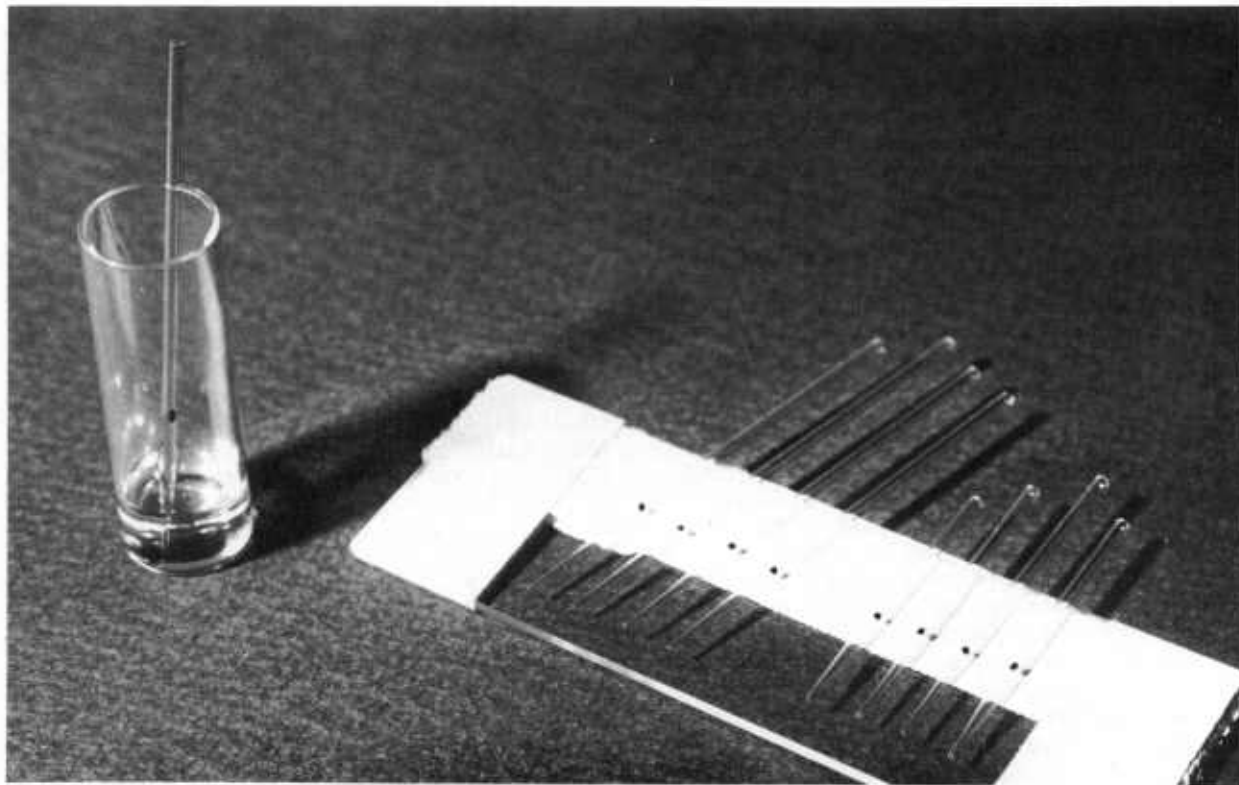
Application of Growth Substances With Capillary Tubes

Small, pointed glass capillaries that hold a required volume of growth regulator solution are useful for introducing minute quantities of the solution into a specific part of intact

⁶ Stokes Tablet Coating Machine, Model 29, F. J. Stokes Machine Company, Philadelphia, Pa.

plants. Draw out glass capillaries about 1–2 mm. outside diameter. Draw out the end of each tube to a point. Make each tube so it can hold more than the volume required. Fill an opsonic pipette (graduations about 3 cm. to each 0.01 ml.) with distilled water. Hold the opsonic pipette horizontally and place the capillary to be calibrated against the orifice of the opsonic pipette. In this way, draw the water into the capillary tube. When the meniscus in the opsonic pipette reaches the required volume, remove the capillary tube, mark the meniscus in the tube with a glass knife or file, and then break the capillary tube at this mark. Touch the capillary tube to filter paper to empty it. Tubes made and calibrated in this way will take up the appropriate volume of growth substance solution. The length of the tubes with equal capacity may vary depending upon the bore of the capillaries.

After allowing the tubes to draw up a known volume of the growth regulator solution, stick them to adhesive tape (sticky side out) on a microscope slide (fig. 40). To apply the regulators, remove a tube from the slide and stick the pointed end of the filled capillary into the appropriate part of the plant.



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FIGURE 40.—Capillary tubes for introducing known amounts of growth-regulating substances into intact plants. (Illustration—after photo by H. W. B. Barlow.)

Direct insertion into a young internode on apple or plum shoots caused damage to that internode, even though there were responses to gibberellins above and below the point injected. Insertion into the petiole was preferable, particularly when the lamina was removed, as it did not induce injury response to the stems.

If one capillary empties at a much slower rate than the majority of the others, remove this one and reinsert it after breaking a small piece off the tip.

This method is particularly valuable when only very small quantities of material are available or when application of measured dosages to particular sites is required. When used in the field, the capillaries remain attached to the plant reasonably firmly, even on windy days.

Reference

Submitted by H. W. B. Barlow, East Malling Research Station, East Malling, Maidstone, Kent, England.

A somewhat similar technique can also be used. Supply the solution of growth-regulating substance to a pedicel or stem of an intact plant, such as tomato or cucumber, through a glass tube, 4–6 mm. in inside diameter tapering to not more than 0.5 mm. in outside diameter. Fill the tapered end of the tube with the growth-regulator solution by capillarity. Plug the large end of the tube with cotton. Insert the tapered end of the tube directly into the pedicel or stem. Support the tube by threads tied around the tube and around a stationary support.

Reference

Homan, D. N. Auxin Transport in the Physiology of Fruit Development. *Plant Physiol.* 39: 982–986. 1964.

Easy Method of Impregnating Dust Carriers With Regulating Chemicals

Weigh the desired amount of carrier (diatomaceous earth or other finely ground inert material) and place it in a container. Dissolve the required amount of regulating chemical in a sufficient amount of ethyl alcohol to make a slurry when combined with the dust carrier. Combine the alcohol solution with the dust (fig. 41). Stir the slurry thoroughly and allow the alcohol to evaporate at room temperature. Mix the impregnated dust thoroughly before applying it to plants.

Useful concentrations of regulating chemicals in dust carriers often range from 500 to 5,000 parts of the chemical in 1 million parts of carrier. Effectiveness of some regulators ap-



PN-304

FIGURE 41.—Impregnating dust carriers with regulating chemicals.

plied in talc dust has been slightly enhanced experimentally by the addition of small amounts of a hygroscopic agent, such as glycerine, to the alcoholic mixture. During evaporation of the alcohol, the hygroscopic agent is incorporated into the dust preparation.

Convenient Method of Dispersing Relatively Insoluble Organic Compounds in Water

Many growth-regulating substances in the form of acids or esters are not readily soluble in water. In dispersing these substances in water, the final mixture may be a suspension, of relatively large groups of molecules of a substance. These particles, however, may be so small that the final mixture is clear and looks, temporarily at least, like a true solution. Upon standing, however, such a suspension of minute particles may aggregate, with the result that the chemical falls out of mixture. The size of the dispersed molecular aggregates, with the growth regulator initially in the mixture and subsequently maintained in the mixture, governs to some extent the amount of the compound absorbed by the plant. The amount of response obtained, therefore, indirectly depends to some extent on the way a relatively insoluble growth regulator is dispersed in a water mixture.

To obtain and maintain even dispersion as long as possible, it is often desirable to use an adjuvant, sometimes termed a "cosolvent."

Such cosolvent should be relatively nonvolatile, should be a solvent for the growth regulator, should have strong surfactant properties, and should be nontoxic to plants at effective concentrations.

Many commercially available surfactants can be used as cosolvents in experimenting with relatively insoluble growth-regulating chemicals. Tween 20, a sorbitol derivative obtainable from the Atlas Powder Company, Wilmington, Del., is used here to illustrate how a cosolvent aids in dispersing a regulating chemical in water and in keeping a regulator finely divided even after the water in the mixture evaporates from the leaf or stem surface.

Measure volumetrically a sufficient amount of the surfactant to make a final concentration of 0.1%. Add the weighed amount of regulating chemical to the surfactant, warm the mixture (not above 50° C.), and stir until the chemical is dissolved in the surfactant. With rapid stirring, introduce the surfactant-growth regulator mixture into a volume of warm water sufficient to give the desired final concentration. If relatively small amounts of chemical and surfactant are involved, use a microbeaker made by cutting off the lower end of a small shell vial. Weigh the required amount of surfactant into the microbeaker and dissolve the required amount of test compound in the surfactant by gently warming and stirring the mixture with a micro glass rod. Place the entire microbeaker containing the mixture into the required amount of water while rapidly

stirring the water. Care should be taken that the surfactant mixture is poured into the water and not the water into the surfactant mixture.

It is impractical to describe here how the many other equally effective surfactants can be used. No discrimination is intended against these other surfactants which include, for example, Tween 80 (Atlas Powder Co.), Carbowax 1500 (Union Carbide Chemicals Co., New York, N. Y.), Triton X 100 (Rohm & Haas Co., Bristol, Pa.), and dimethyl sulfoxide, known as DMSO, (Crown Zellerbach Corp., Camas, Wash.).

For further information regarding surfactants see:

Schwartz, A. M., and Perry, J. W. *Surface Active Agents—Their Chemistry and Technology*. v. 1. 579 pp. Interscience Publishers, Inc., New York, and Interscience Publishers, Ltd., London. 1949.

Schwartz, A. M., Perry, J. W., and Berch, Julian. *Surface Active Agents and Detergents*. v. 2, 839 pp. Interscience Publishers, Inc., New York, and Interscience Publishers, Ltd., London. 1958.

Segment Cutters

Segments of plant parts, such as coleoptiles and stems, are used to detect and measure plant growth-promoting and growth-inhibiting substances. Various instruments have been devised so uniform segments of required lengths can easily be made from these and other plant parts. Diagrams of 3 easily constructed cutters are shown in fig. 42.

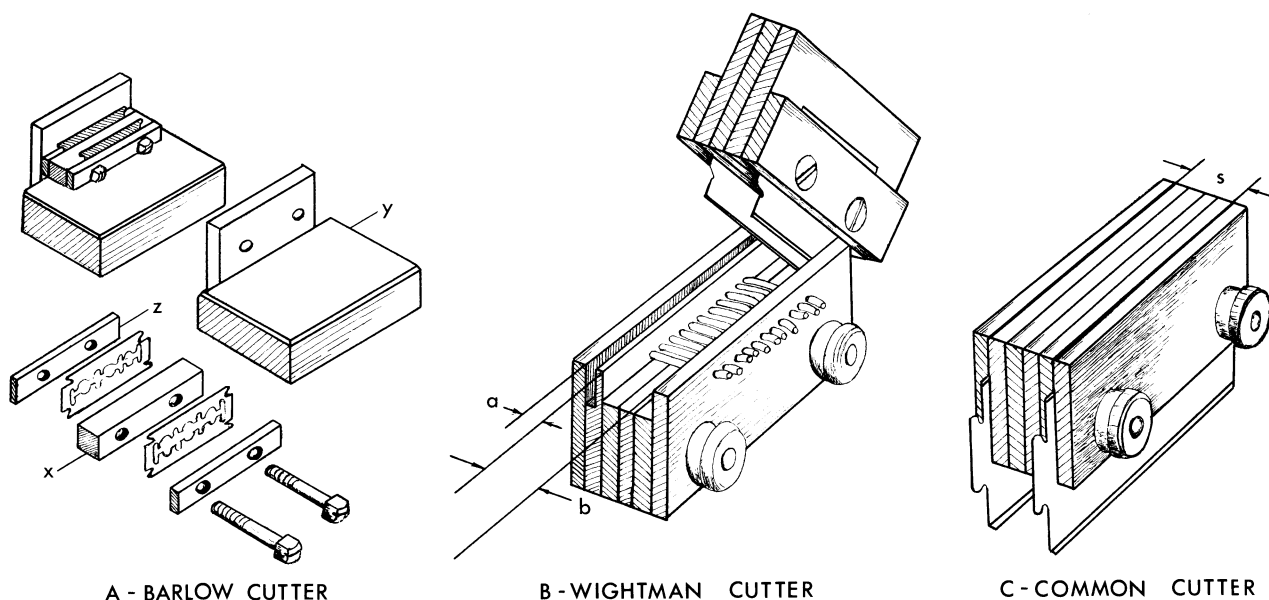


FIGURE 42.—Segment cutters. (A, Diagram—courtesy of Journal of Experimental Botany; B, diagrammed from photo by F. Wightman.)

Barlow cutter (fig. 42, A): Make the separator bar (x) wide enough to hold the razor blades the required distance apart. Construct several of these interchangeable separator bars of required widths, if segments of different lengths are needed. Make separator bar (z) wide enough to hold the razor blade nearest the guide plate 3 mm. from the guide plate. Base plate (y) can be made of either wood or metal, the other parts of aluminum or brass. To make coleoptile segments with this cutter sever the coleoptiles from the seeds with a scalpel. Place the coleoptiles in rows on wet microscope slides, keeping them in the chronological order in which they were cut. Arrange the coleoptiles at right angles to, and with their tips coincident with, one edge of the slide. Place the slide against the zero line of an aluminum grader scribed at 5-mm. intervals. Place coleoptiles with bases falling between any 2 adjacent lines in one length grade, maintaining chronological order. Align the tips with the slide as before. Using a separator bar that holds the blades 10 mm. apart, invert the slide containing coleoptiles and place it on the blades of the cutter. Keep the tips against the guide plate and then exert a gentle downward pressure. Remove the unwanted 3-mm. tips thus cut from the coleoptiles by passing a needle between the guide plate and the nearby blade; also remove the unwanted basal ends cut from the coleoptile. Remove the cut segments by inserting the needle between the blades and under the coleoptile segments. While pressing the segments gently against the glass surface, lift off and invert the slide. Retain only the segments that are completely filled with leaf tissues. (See "Wheat coleoptile straight growth method," p. 36.)

Wightman cutter (fig. 42, B): Construct the cutter of aluminum or brass so the distance (a) is 3 mm. The distance (b) can be changed to make any desired length of segment by varying the thickness of the separator plates in the part of the instrument holding the coleoptile and also the width of the plates separating the razor blades. In making segments, discard the 3-mm. tips and the basal ends of the coleoptiles. Remove the primary leaves by threading the measured segments onto fine glass capillaries, 2 segments per capillary, and then allow the segments to float on distilled water in a petri dish until required to start the experiment. Do not allow this pretreatment period to exceed 3 hr. (See "Wheat coleoptile straight growth method," p. 36.)

Common cutter (fig. 42, C): Construct aluminum or brass razor blade holder as illustrated. Adjust the blades to the desired distance apart

(s) by varying the width or number of separator plates used.

Device That Delivers Measured Amounts of Regulating Chemical

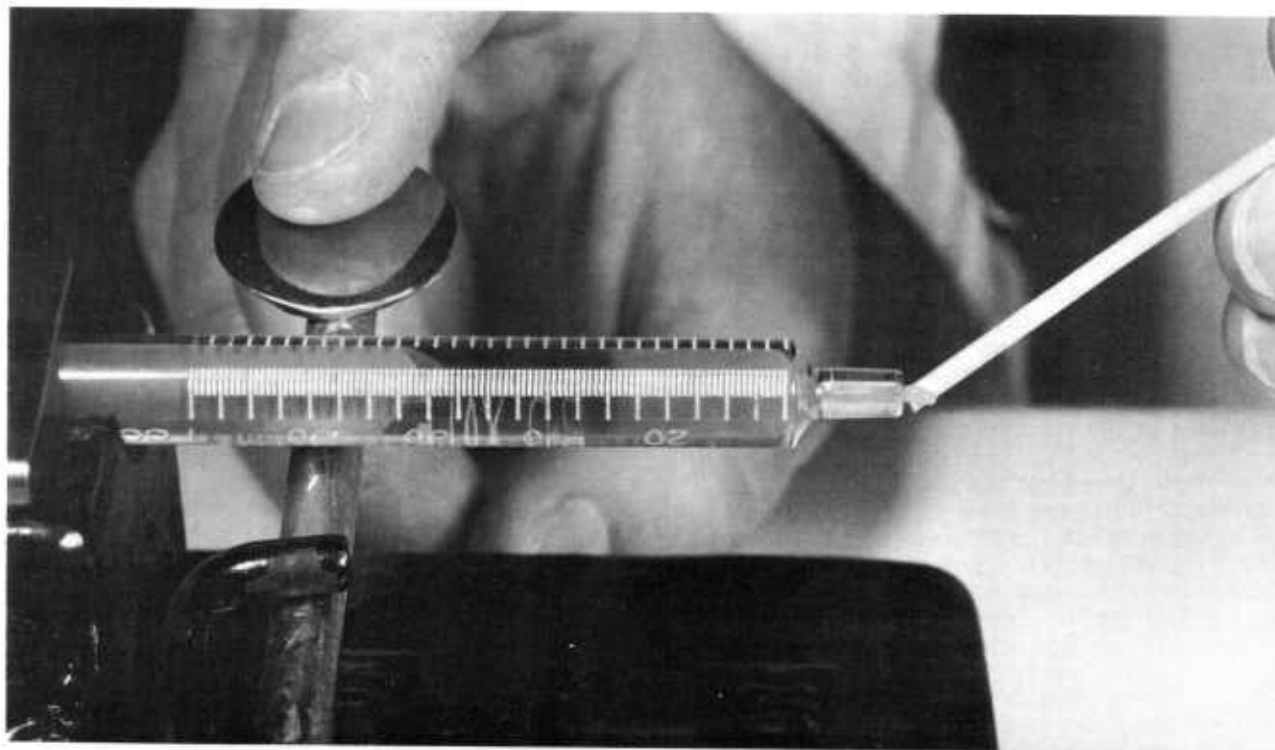
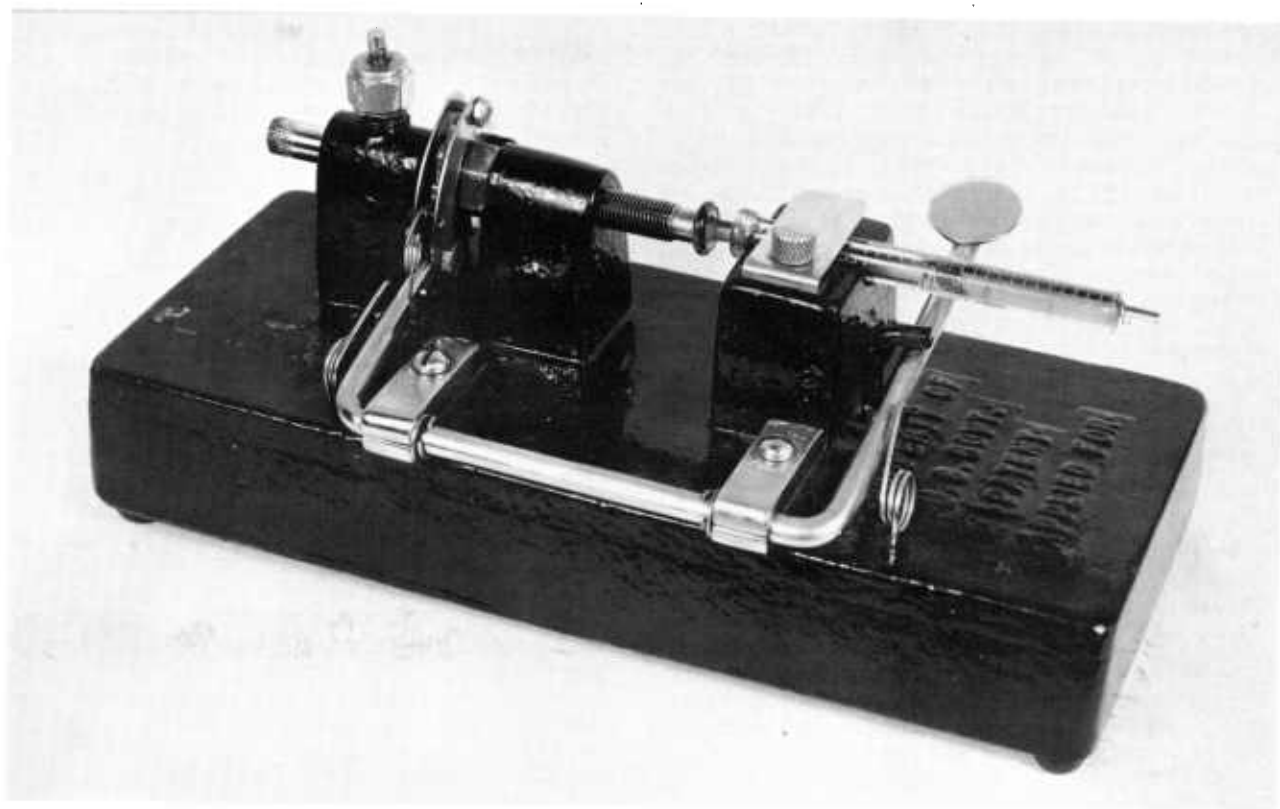
The microinjector shown in fig. 43 delivers measured volumes of lanolin paste containing a regulating chemical (fig. 43, *upper*). Prepare a mixture of lanolin and the regulating chemical. While the mixture is melted, draw the syringe about one-third full; then allow the mixture in the syringe to solidify. Fasten the syringe in the clamp and operate the lever until a uniform amount of the paste is delivered with each stroke. Collect portions of desired volume (regulated by the number of strokes used) on the end of a toothpick or other disposable applicator (fig. 43, *lower*) and apply the paste quantitatively to leaves or stems. Determine the weight of paste delivered per stroke by first weighing a piece of aluminum foil, then collecting on the foil the amount of paste ejected after one stroke, and reweighing the paste and foil. Repeat the procedure 5 to 10 times; then calculate the average number of micrograms of growth-regulating chemical delivered per stroke.

Construction of this microinjector is described by C. C. Roan and Shizuko Maeda in "A microdevice for rapid application of toxicants to individual insects." publication No. ET-306 of the former Bureau of Entomology and Plant Quarantine, U.S. Department of Agriculture, 1953.

Direct Measurement of Volatility⁷

Prepare an alcohol solution that contains 10,000 p.p.m. of the growth-regulating chemical to be tested. (This technique is not suitable for measuring the volatility of hygroscopic compounds.) Weigh a sheet of tinfoil 3×6 cm. (Aluminum foil is not satisfactory for this test.) Dip the tinfoil into the alcohol solution and allow the excess to drain off for a few moments. Immediately attach the tinfoil to the beam of a balance so that the foil swings freely. Record the combined weight of the tinfoil, alcohol, and growth regulator at frequent intervals within the next few minutes to determine when the relatively rapid rate of weight

⁷ Carelessness in the use or disposal of highly volatile regulating chemicals can result in contamination of an entire laboratory or greenhouse, thus causing loss of valuable plants and space.



PN-803, A and B

FIGURE 43.—Device that delivers measured amounts of regulating chemicals.

loss due to evaporation of alcohol changes to a slower rate of weight loss, which in most instances represents the evaporation of the growth regulator. Weigh the remaining growth regulator and the tinfoil at intervals to determine the rate of evaporation of the growth regulator. Pieces of thin glass about 25 square cm. in area may be used in place of the tin foil. For other methods of measuring volatility of plant regulators, see "Volatility of plant regulators," p. 90.

Reference

Anderson, W. P., Linder, P. J., and Mitchell, J. W. Evaporation of Some Plant Growth Regulators and Its Possible Effect on Their Activity. *Science* 116: 502-503. 1952.

Fractionated Lanolin as a Carrier for Growth-Regulating Compounds

Lanolin is an excellent carrier for growth-regulating substances, provided the compound is first dissolved in a suitable cosolvent and then mixed with the lanolin. Lanolin, however, remains relatively solid or viscous at ordinary room temperatures, even though there may be heat from a nearby lamp being used for illumination when treating small areas on plants with such a mixture. By fractionating lanolin, a fat with slightly more fluidity than that of lanolin can be obtained. This fraction usually becomes very fluid when applied under a lamp, thus affording excellent contact between the fractionated lanolin mixture and the surface of the plant. Use the following procedure to make this lanolin fraction.

Warm *carefully* on a steam bath 10 g. of anhydrous lanolin (U.S.P. *Adeps lanae*) dissolved in acetone (6 parts of acetone by weight to 1 part of lanolin) until the temperature of the mixture reaches about 50° C. The steam bath is preferred because of the flammability of acetone, which boils at 56° C. However, proximity to flame or electrical contacts that might spark is to be avoided since acetone flashes at -15° C., far below its boiling point. Stir the mixture carefully until the lanolin is dissolved. Allow the solution to cool to room temperature (25° C.) overnight. Cover the beaker with foil to retard evaporation during this period. Recover the solution from the precipitate by any convenient method such as filtration, decantation, or centrifugation followed by decantation. Warm the solution on a steam bath to evaporate the acetone and recover a fraction that is slightly more fluid than the lanolin itself. Lower precipitation temperatures will produce a more fluid fraction but with reduced yield.

Investigators may also wish to determine the possible usefulness of another fraction of lanolin known as Viscolan, available from the American Cholesterol Products, Inc., Amerchol Park, Edison, N. J. 08817.

Reference

Method submitted by E. A. Wilder, S. C. Johnson and Son, Inc., Racine, Wis.

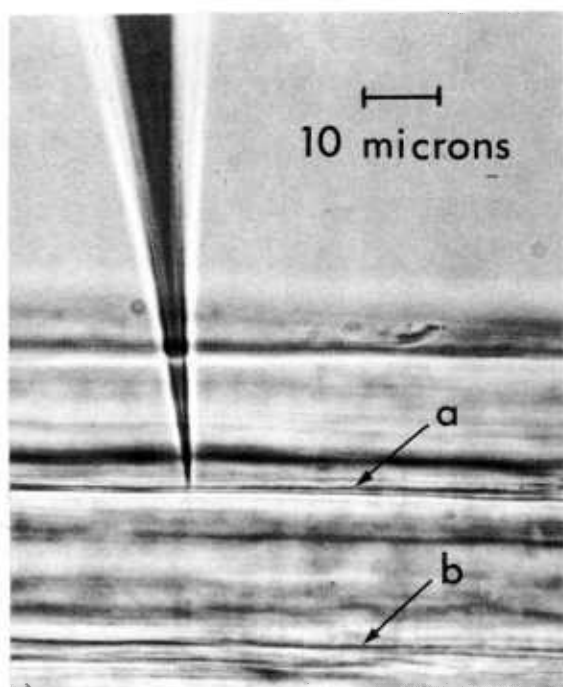
Injecting a Regulating Substance or Hormone Into a Single, Living Cell

Intracellular responses to regulators and hormones can be observed and photographically recorded during and following injection of the accelerator or inhibitor into an individual cell. In addition to plant material to be studied, the following equipment is needed: capillary hard glass tubing (such as Pyrex or Kimax), a pipette puller, micromanipulators, high-powered microscope, injection equipment, water-immersion objective with which a magnification of 400× can be obtained, still camera for photomicrographs, and equipment for cinematography if the response of living cells is to be studied in terms of protoplasmic movements and changes as the responses progress.

Prepare sections of living plant tissue to be studied. (See "Freehand sections for studying cell responses to regulators," p. 120.) Mount these sections in tapwater on a microscope slide without a cover slip. Use two hypodermic needles to hold the plant section against the surface of the slide, both needles being held by a single micromanipulator. With the beveled side of the 2 needles facing downward, press the edges of the section against the glass while leaving the cells to be studied uninjured.

Make a micropipette with a tip that is barely visible when magnified 400×. A pipette of this diameter generally has an opening that is 0.5 micron or less in diameter. Such an opening is not visible at this magnification. Fill the micropipette by capillary action by immersing the tip for several minutes in the solution to be injected. Join the micropipette to a microinjection apparatus mounted on another micromanipulator.

Position the micropipette and section under 35× magnification. Figure 44 shows tip of micropipette at cell wall (*a*) of fiber cell (*a-b*). Switch to the water-immersion objective (400×) and inject the cell by inserting the micropipette through the cell wall and for a very short distance into the cytoplasm. Transfer sections containing successfully injected cells to tapwater contained in a slide chamber. (See "Slide chamber for the study of living



BN-28953

FIGURE 44.—Micropipette touching surface of a fiber cell (a) before puncture of cell wall and injection of growth-regulating substance; (b), wall on opposite side of fiber cell.

cells," p. 123.) Observe the cells through the cover slip and record the responses photographically.

A droplet of castor oil can be injected into some kinds of cells without apparent injury to the protoplasm. Dissolve the required amount of the test substance in the oil and inject it as a single droplet into the cell to be studied.

Various pipette pullers are commercially available, including the following: Livingston Micropipette Puller made by Otto K. Hebel, 80 Swarthmore Avenue, Rutledge, Pa.; one made by E. Leitz, Inc., 468 Park Avenue South, New York, N. Y. A Livingston Micropipette Puller was used in developing the method described above.

Reference

Method submitted by J. F. Worley, Agricultural Research Service, U.S. Department of Agriculture.

Injecting Substances Into Trees

Growth regulators, antibiotics, or other substances can be injected into trees through holes

bored in their branches or trunks. Effects of these substances on growth, fruit set, fruit drop, longevity, and on systemic disease control can be studied. With a specially made tool (fig. 45, a), the female part of a plastic quick connector (fig. 45, b) is driven into a hole previously made with a hand drill. The male part of the quick connector, with rubber tubing and plastic bottle attached to one end, is fitted into the female part already in the tree (fig. 46, p. 107). Small quantities of test solution can be forced into the bottle through a small hole with a large hypodermic syringe and allowed to flow by gravity into the tree. With large trees, 2 or more injection sites may be used with containers with a capacity of 1 liter or more.

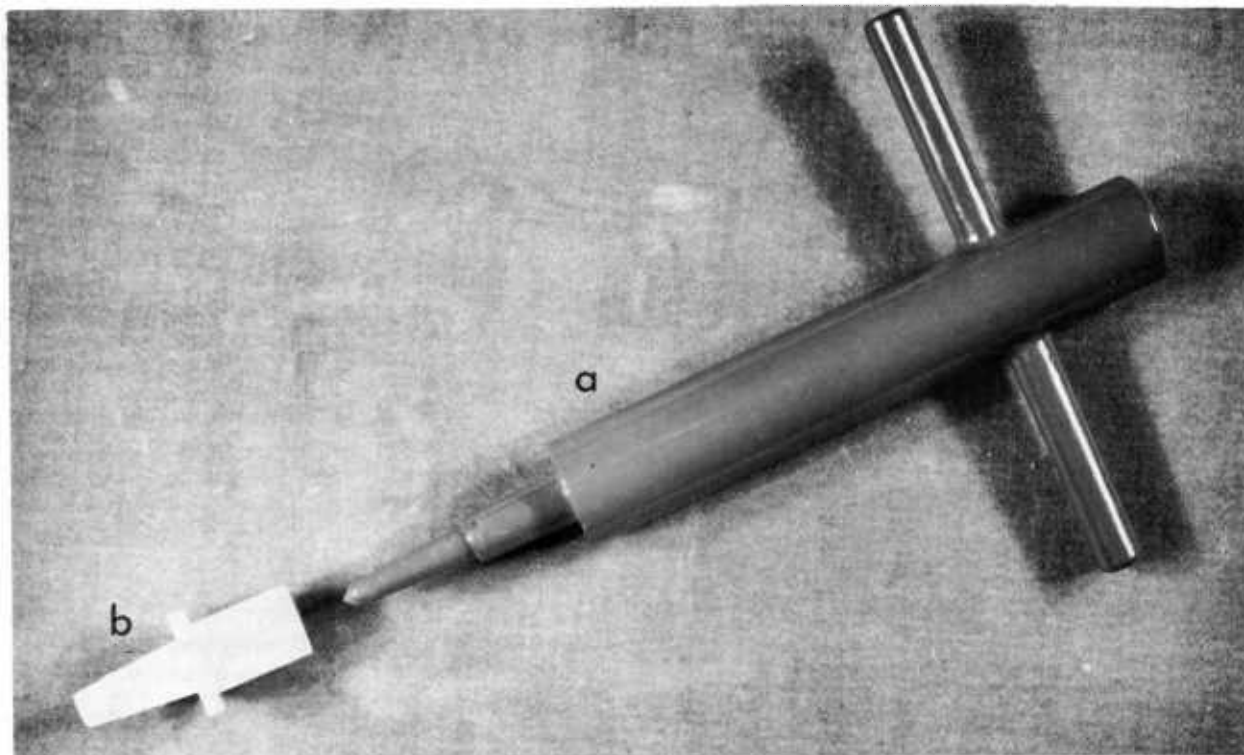
Reference

Method submitted by H. L. Keil, Agricultural Research Service, U.S. Department of Agriculture.

Introduction of Chemicals Directly Into Xylem Elements in Stems of Herbaceous Plants

Small amounts of radioactively tagged compounds have been introduced directly into xylem elements to study transport of the substances. The method described here applies to young bean plants, but the technique should also be useful for studying translocation in other herbaceous plants. Carry out a preliminary microscopic examination of the stem anatomy of the test plant chosen to become acquainted with the location of xylem elements leading to a primary leaf. To do this, cut freehand cross sections through the first internode of several of the plants at a level midway between the first and second nodes. Observe tracheary elements that lead to one primary leaf and note their position in relation to the outside contour of the stem.

Select a plant comparable to those examined microscopically and determine the exact location on the first internode to which a droplet should be applied so the droplet will be directly outside of the tracheary elements leading to the primary leaf. At this point on the stem, apply a 0.005-ml. droplet of water containing the plant regulator to be tested. Make a tool to sever some of the tracheary elements leading to the leaf by forming a wedge-shaped chisel approximately 0.8 mm. wide on the end of a length of fine steel wire. This provides a sharp cutting edge. Immerse the chisel in the droplet and, far as the pith (fig. 47), thus severing, in the with slight pressure, insert it into the stem as case of young plants, the primary tracheary elements connected with the leaf. When the instrument is withdrawn, the droplet is sucked into



BN-28942

FIGURE 45.—Special tool (a) used to insert quick connector (b) into tree.

the plant through the opening into the stem. The rapidity of droplet intake may vary from a few seconds to a minute or more. The investigator may wish to classify the plants on the basis of time required to absorb the droplet and thus include the rate of absorption along with other factors involved. The absorbed liquid is moved upward from the place where the xylem elements are severed into the primary leaf. Repeat the procedure to obtain the required number of treated plants. After the desired interval following treatment, determine the distribution of the radioactivity by means of radioautographs. (See "Radioautograph method," p. 87.)

Reference

Linder, P. J., and Mitchell, J. W. Rapid Transport of Alpha-Methoxyphenylacetic Acid Introduced Directly Into the Water Stream of Bean Plants. *Bot. Gaz.* 121: 139-142. 1960.

not injure plants. It makes close contact with the plant surface, and regulators, when mixed with lanolin, diffuse readily from a thin layer of the paste into the plant. Place a weighed amount of lanolin in a vial and immerse the vial in warm water to melt the lanolin. Weigh out the finely powdered or liquid regulator and stir it thoroughly into the lanolin until the mixture becomes semisolid. Apply the mixture to stems or leaves of broadleaf plants with a toothpick or other disposable applicator.

In the case of grass leaves, use a glass rod 2 mm. in diameter to spread the lanolin mixture in a thin, even layer completely covering a portion of the upper surface of the leaf for a distance of 1 cm. To do this, draw the rod from a position near the base of the leaf toward the tip. With practice, uniform amounts of compound can be applied rapidly by this means.

Lanolin as a Carrier for Regulating Chemicals

Lanolin is widely used as a carrier for regulating chemicals (fig. 48). Applied in small amounts, this semisolid, fatty substance does

Movement of Regulating Substances Through Plant Membranes or Tissues

A simple apparatus has been used for studying movement of substances through plant



BN-28941

FIGURE 46.—Injecting a regulator into tree with plastic bottle, rubber tubing, and quick connector.

membranes or layers of plant tissue (fig. 49). Fill a screwcap vial (approximately 9 mm. in inside diameter at the open end) to about four-fifths of its capacity with a solution of the compound to be tested. Cut off the closed end of the cap, thus making a threaded collar. This collar must fit the threaded area on the screwcap vial. It must not be wider than the threaded area. Place the plant membrane over the open end of the vial and screw the collar completely on the threaded area of the screwcap vial to fasten the membrane tightly across the opening. Insert the vial through a hole in a disk of relatively thick sheet rubber, a support for the screwcap vial. Make the diameter of the hole in the rubber slightly smaller than the outside diameter of the vial. Place a measured amount of distilled water

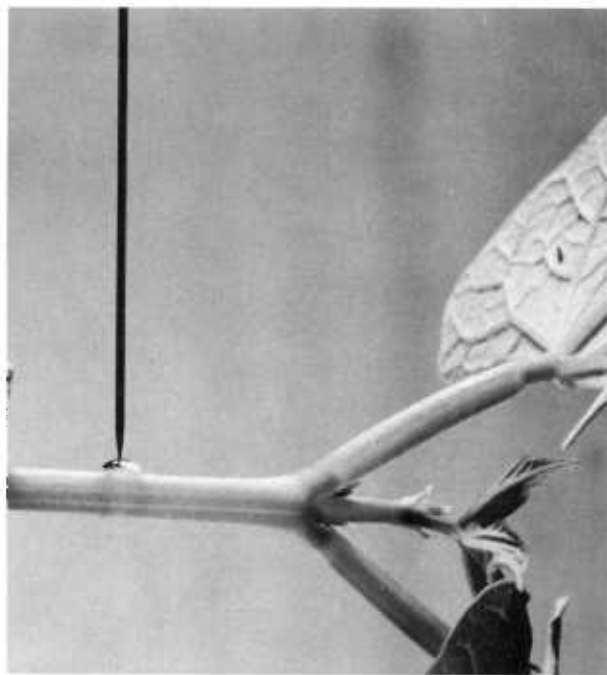
in a shell vial of greater inside diameter than that of the screwcap vial. Invert the screwcap vial with its membrane attached and adjust its position in the rubber support so that the membrane is below the surface of the water in the shell vial. Allow the substance being tested to diffuse through the membrane for the required length of time (2, 4, or 8 hr.). Remove the screwcap vial, stopper the shell vial, and subsequently determine the concentration of the substance that has diffused through the membrane by appropriate bioassay methods, colorimetric methods, or the use of radioactively tagged compounds.

Reference

Szabo, S. S., and Buchholtz, K. P. Penetration of Living and Non-Living Surfaces by 2,4-D as Influenced by Ionic Derivatives. *Weeds* 9: 177-184. 1961.

Plastic Culture Vessel ("Plasticon") for Small Plants

Plastic culture vessels (fig. 50, p. 110) are useful for supporting small plants with their roots suspended in nutrient solutions or liquids containing chemicals to be tested. Construct a



BN-28940

FIGURE 47.—Severing tracheary elements in stem of young bean plant with tip of metal chisel immersed in droplet containing plant regulator.

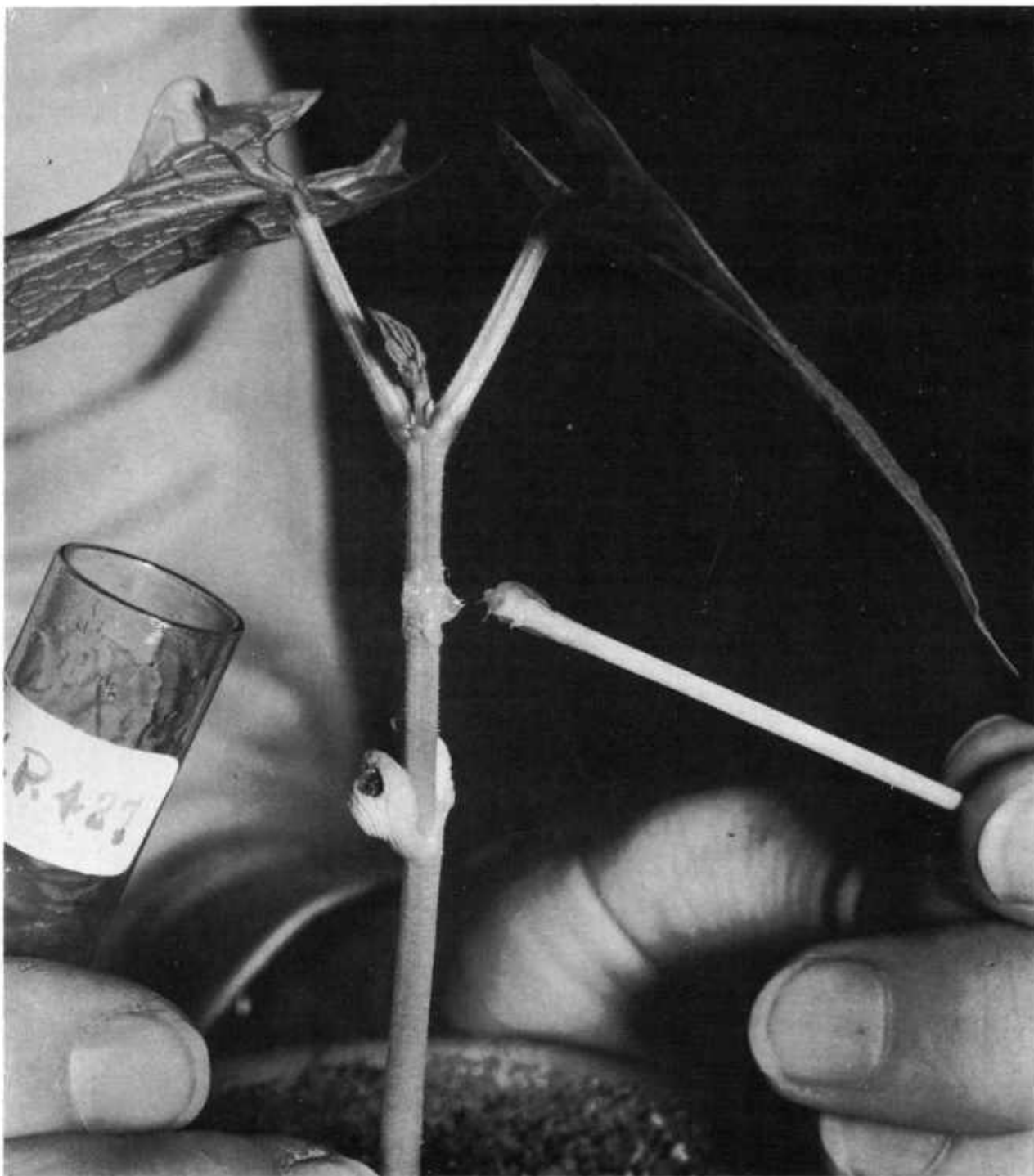


FIGURE 48.—Applying lanolin containing a regulating chemical to stem.

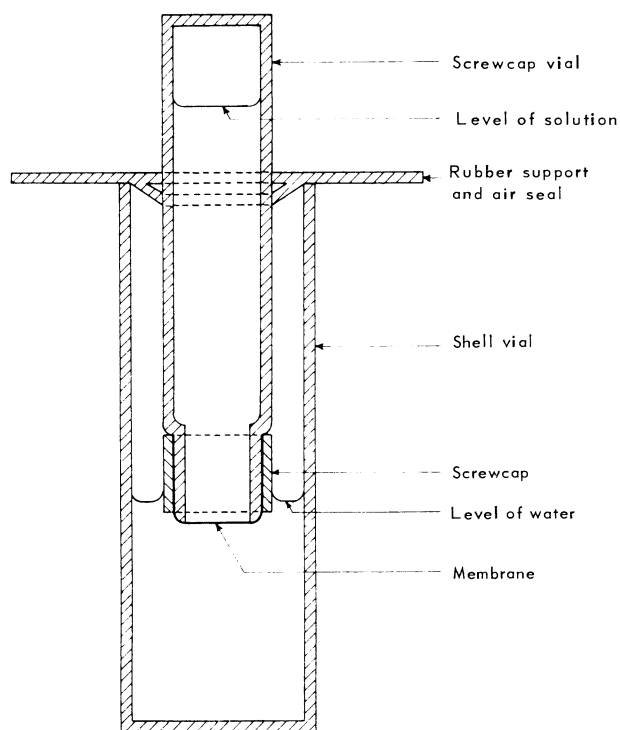


FIGURE 49.—Apparatus for studying movement of regulating substances through membranes or tissues. (Photo—courtesy of Weeds.)

plastic vessel with an upper and lower part, using Plexiglas (methyl methacrylate synthetic plastic) (fig. 51). Close the lower part with Plexiglas, forming a cylindrical reservoir about 8 cm. in diameter. For the upper part, make a disk slightly greater in diameter than the cylinder of the reservoir and seal a hollow Plexiglas cylinder of smaller diameter in the center of this disk as shown. Stretch a netting of nylon across the lower end of this small cylinder and seal the netting as shown. Use dissolved plastic to seal the various pieces together. Place the required number of seeds on the netting and adjust the volume of liquid as illustrated. Construct a cylinder of opaque paper to fit over the reservoir or apply aluminum paint to partly exclude light and prevent growth of algae.

To aerate the liquid in the plasticon, construct a manifold using T-tubes connected with plastic tubing. Attach pieces of plastic tubing to "bubblers," such as are used to aerate an aquarium, and insert the tubing through a hole drilled in the top of the plasticon. Connect the plastic tubing with the outlets of the manifold. Immerse the bubblers in the liquid with the plasticon. Connect one end of the manifold to an aquarium pump and leave the other end of the manifold open but with a piece of plastic tubing and pinch

clamp attached. Force compressed air through the bubblers with the pump. Adjust the rate of airflow by means of screw clamps on each manifold outlet and the pinch clamp at the end of the manifold.

Reference

Hagborg, W. A. F. The Effect of Antibiotics on Infection of Wheat by *Xanthomonas translucens*. *Canad. Jour. Microbiol.* 2: 80–86. 1956.

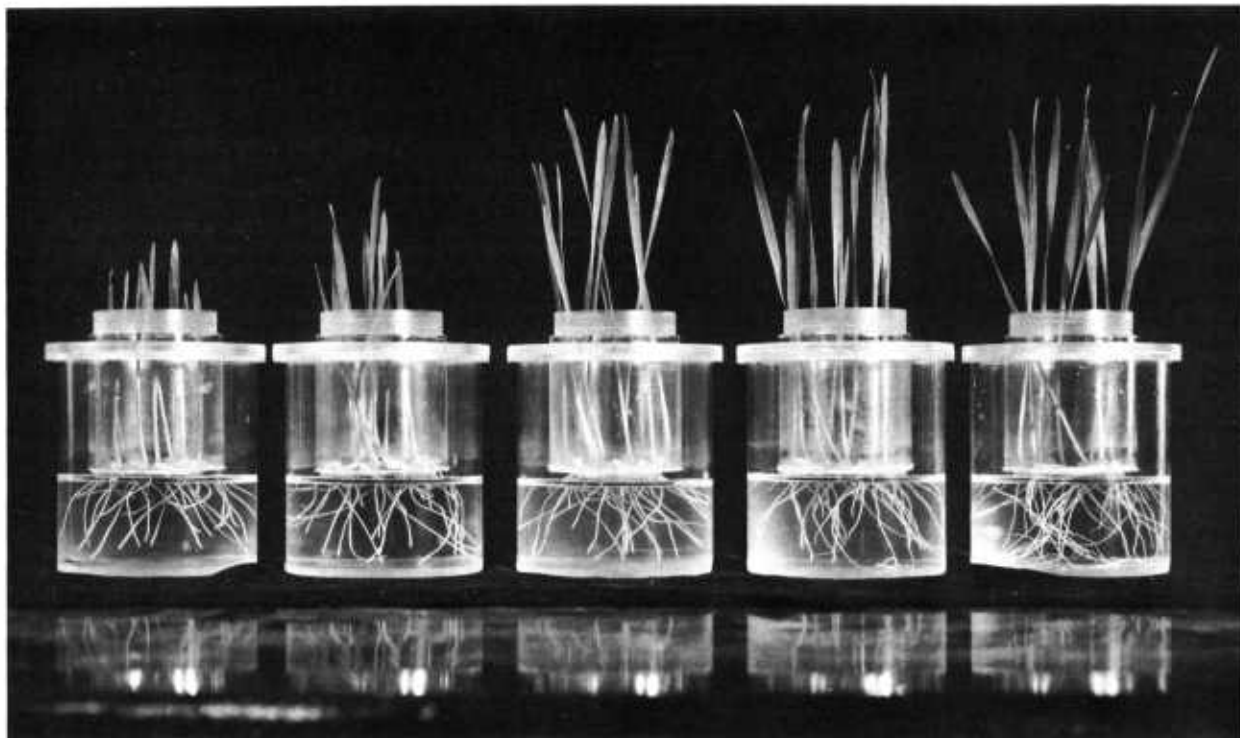
Simulated Spray-Droplets

Droplets of different sizes and various patterns of distribution can be obtained to simulate spray-droplets. Some investigators use a small glass loop to place droplets of relatively uniform size on leaves. With the aid of a flame, draw out a glass rod so that it forms a thin thread about 0.5–0.75 mm. in diameter. Break the thread of glass and with very little heat bend the end of the small rod so that a loop 2–3 mm. in diameter is formed. Dip the loop into an aqueous mixture of the regulating chemical. Transfer the liquid that adheres to the loop onto the surface of a leaf by touching the loop to the desired area (fig. 52).

Technique for Studying Absorption and Translocation of Regulating Substances by Aquatic Plants

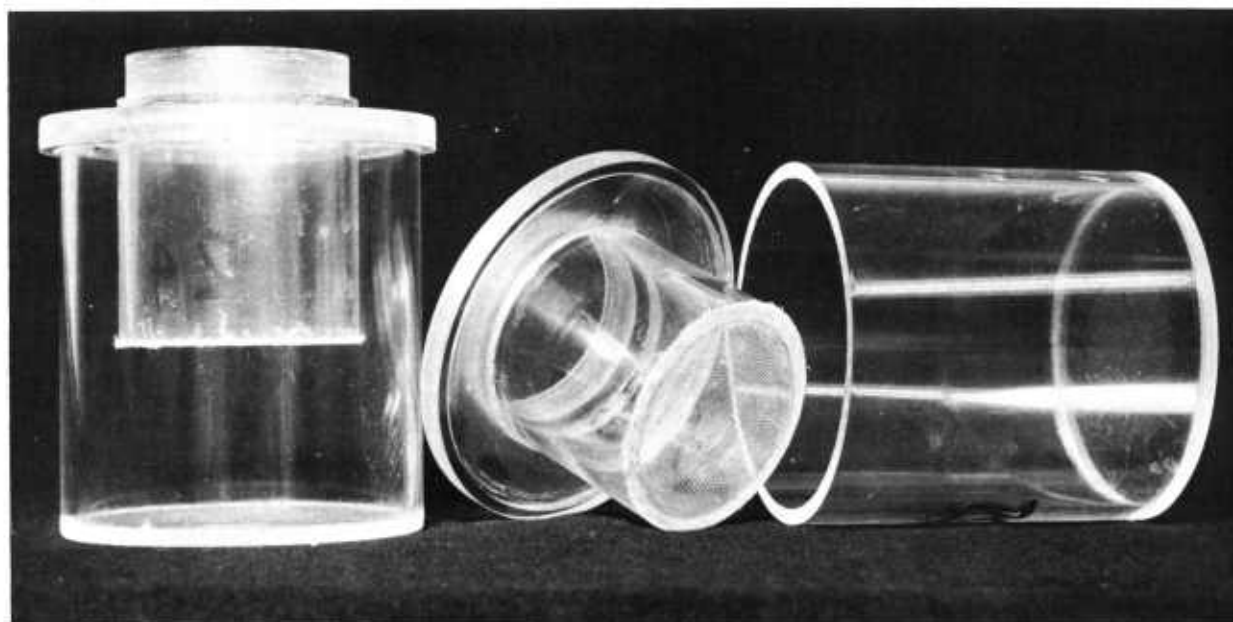
The following technique has been used to separate different parts of submersed water plants so absorption and translocation of a growth-regulating substance can be studied.

Place the stem (7.5 cm. long) of a polyethylene funnel, with a capacity of 750 ml. of liquid, through a hole in a No. 12 rubber stopper (fig. 53). Thread a water plant, such as sago pondweed (*Potamogeton pectinatus* L.), through the stem of the funnel, allowing the roots to protrude from the stem. Lightly block the hole in the funnel stem with one finger and fill the stem of the funnel with melted eicosane (fig. 53, e). When the surface of the eicosane has solidified, immerse the plant and funnel with the eicosane plug in cold water to solidify the entire plug. Place 250 ml. of the solution containing the growth-regulating substance being tested in a wide-mouth bottle that can be tightly closed with the rubber stopper. Submerge the roots of the plant in this solution and fit the stopper snugly into place. Pour 500 ml. of water into the funnel, thus submersing the upper part of the plant. Use a 5–7 mm. glass tube leading from inside the bottle through the stopper to a level above the funnel as a vent to accommodate expansion or contraction of the liquid in the closed



BN-28939

FIGURE 50.—Plastic culture vessels ("plasticons") for small plants. (Photo—courtesy of Canadian Journal of Microbiology.)



BN-28938

FIGURE 51.—Parts of a plasticon. (Photo—courtesy of Canadian Journal of Microbiology.)



PN-809

FIGURE 52.—Simulating spray-droplets on a leaf.

part of the system. If absorption and translocation from the top of the plant to the root is to be measured, place a solution of the growth regulator in the funnel. Fill the bottle with water. After an appropriate treatment period, remove the stopper and funnel, pour the liquid from the funnel, and separate the roots from the top by severing the stem within the eicosane plug. Blot the plants dry after washing them in running water and arrange them on blotting paper. Freeze the plants with solid carbon dioxide and allow them to dry. Prepare radioautographs to show absorption and translocation based on the distribution of the isotope. Assay aliquots of the liquid in the top and bottom of the apparatus at the end of the experiment to be sure that leaks did not develop. Do this by evaporating aliquots of the liquid in planchets and then measuring any radioactivity present with a conventional gas-flow counter.

Reference

Frank, P. A., and Hodgson, R. H. A Technique for Studying Absorption and Translocation in Submersed Plants. *Weeds* 12: 80-82. 1964.

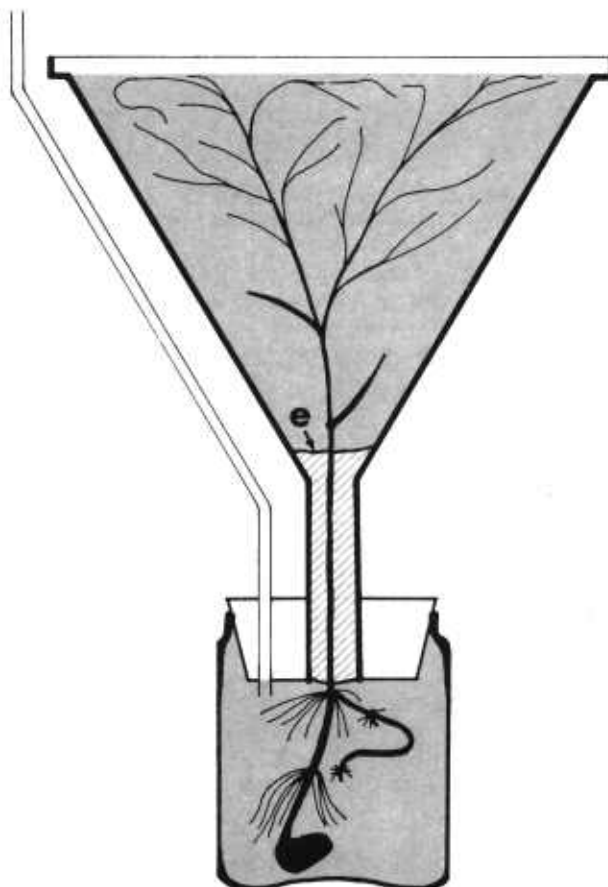


FIGURE 53.—Diagram of apparatus and aquatic plant arranged to study absorption and translocation of regulating substances. (Diagram—courtesy of Weeds.)

Use of Dwarf Corn to Detect and Identify Gibberellins and Gibberellin-Like Substances

Dwarfism in some plants can be partly or wholly overcome by the addition of gibberellins or gibberellin-like substances. Some dwarf mutants, therefore, are useful in detecting and classifying regulating substances that induce elongation and growth of these plants. Six single-gene mutants of maize (*Zea mays* L.) are especially useful for studying gibberellins and gibberellin-like substances. These are: Anther ear-1, Dwarf-(5232), Dwarf-1, Dwarf-(8201), Dwarf-(4963), and Dominant-dwarf. The first 4 of these mutants are relatively sensitive to gibberellic acid, the fifth responds slightly to this compound, and the sixth does not respond to the acid. Since the response of these mutants to growth-accelerating substances varies, all 6

mutants can be used as a composite bioassay technique in studying gibberellins and plant hormones. Thus, a wide spectrum of response can be obtained by considering the response pattern of all 6 mutants as a unit characteristic of the growth-accelerating activity of the compound.

A few (about 25–50) seeds of the mutant dwarf corn alleles in this experiment can be obtained for propagation from E. P. Patterson, Department of Agronomy, College of Agriculture, University of Illinois, Urbana 61803. The recipient of these seeds will need to increase the seed stock of each dwarf if he wishes to have experimental amounts.

One way of applying the test compound is to place a measured amount of solution containing the chemical and an appropriate wetting agent to the upper surface of the first leaf of the germinating plant. Repeat the treatment at 2- to 3-day intervals using new leaves as they develop during the following weeks. Compare variations in growth rate due to treatment.

References

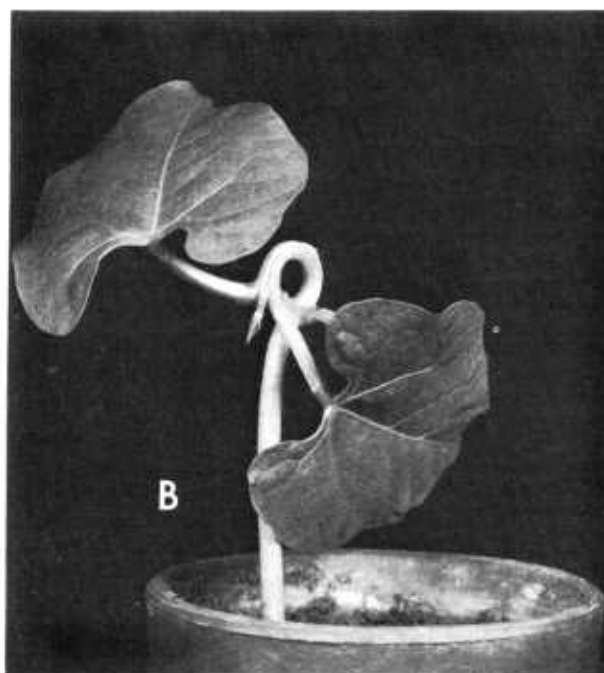
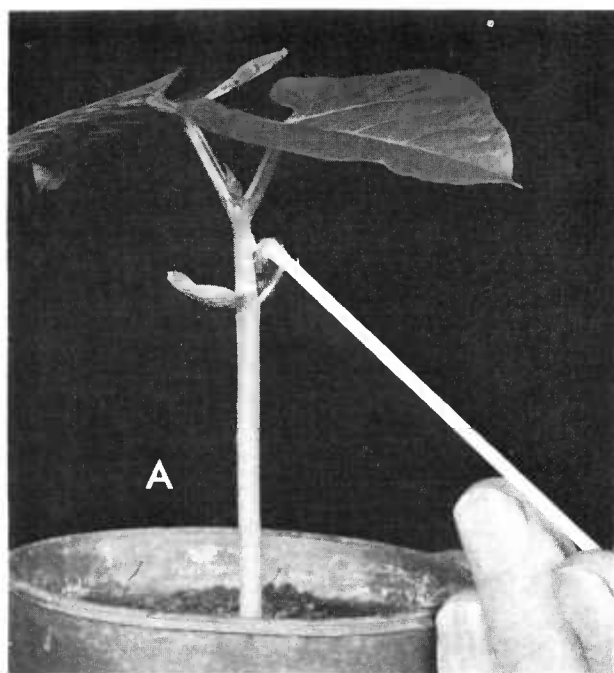
- Phinney, B. O. Growth Response of Single-Gene Dwarf Mutants in Maize to Gibberellic Acid. *Natl. Acad. of Sci. Proc.* 42: 185–189. 1956.
Brian, P. W., and Hemming, H. G. The Effect of Gibberellic Acid on Shoot Growth of Pea Seedlings. *Physiol. Plant.* 8: 669–681. 1955.

GROWING PLANTS

Highly Sensitive Test Plants

Some plants respond quickly to regulating chemicals. Young rapidly growing plants are generally more sensitive to these chemicals than are more mature plants. Young bean, cucumber, and sunflower plants are among the more sensitive, and these are especially useful since they can be quickly and easily grown to a size that is suitable for tests with regulators. Plant

the seeds in composted soil; keep the soil uniformly moist and at a temperature of not less than about 26° C. until the seedlings appear above the surface of the soil; then reduce the night temperature to about 21°–24° C. About 5–8 days is usually required for such plants to grow to a useful size. Application of the regulator in a suitable carrier, such as lanolin, to the stem or leaves of these test plants (fig. 54,



PN-301, A and B

FIGURE 54.—A, Applying regulating chemical to sensitive plant; B, growth response to chemical.

4) will often induce a marked growth response within a few hours or during the following overnight period (fig. 54, B).

References to Methods of Maintaining Constant Relative Humidity

Plant scientists working with regulating substances are sometimes concerned with maintaining known, constant relative humidities. Solutions of inorganic salts, sulfuric acid, bases, or carbohydrates have been commonly used for this purpose. The following references contain complete, reliable data concerning methods of maintaining constant relative humidities.

Carbohydrates

(1) Grover, D. W., and Nicol, J. M. The Vapor Pressure of Glycerin Solutions at 20°. Soc. Chem. Indus. Jour. 59: 175-177. 1940.

Methods of preparing solutions having relative humidity values from 98 to 27 percent at 20° C. by varying the percentage by weight of glycerol are described. Refractive indices for these solutions are given so that by using a refractometer the exact amount of glycerol can be determined. Relative humidity is substantially independent of temperature. These and other data have been redrawn. Sharpf, R. F. A Compact System for Humidity Control. Plant Dis. Rptr. 48: 66-67. 1964.

(2) Shaw, L. Intercellular Humidity in Relation to Fire-blight Susceptibility in Apple and Pear. New York (Cornell) Agr. Exp. Sta. Mem. 181. 40 pp. 1935.

Values for relative humidities between 93.4 and 99.9 at 25° C. are listed for 31 sucrose solutions ranging in molality from 0.1 to 3.0.

Inorganic Salts

(3) Carr, D. S., and Harris, B. L. Solution for Maintaining Constant Relative Humidity. Jour. Indus. Engin. Chem. 41: 2014-2015. 1949.

Relative humidities ranging from 27.4 to 85 which are maintained by 12 different saturated salt solutions at temperatures from ambient to 90° C. are given.

(4) Winston, P. W., and Bates, D. H. Saturated Solutions for the Control of Humidity in Biological Research. Ecology 41(1): 232-237. 1960.

This is a very extensive listing of the relative humidity values of saturated salt solutions at temperatures from 5° to 50° C. at 5° intervals with their properties and methods of preparation. Drying agents that maintain relative humidities of less than 1 to 5.6 percent at 25° C. are also listed. Excellent bibliography.

Sulfuric Acid

(5) Greenewalt, C. H. Partial Pressure of Water out of Aqueous Solutions of Sulfuric Acid. Jour. Indus. Engin. Chem. 17: 522-523. 1925.

The water vapor pressure of concentrated H₂SO₄ solutions (62 to 96%) are plotted as a function of the reciprocal of the absolute temperature.

(6) Wilson, R. E. Humidity Control by Means of Sulfuric Acid Solutions, With Critical Compilation of Vapor Pressure Data. Jour. Indus. Engin. Chem. 13: 326-331. 1921.

Solutions maintaining relative humidities from 0 to 100 percent at 25° C. and their densities are plotted as a function of the percentage H₂SO₄. A method is given for calculating the relative vapor pressure at any temperature that, however, changes very little over a 5-10° C. range.

Bases

(7) Buxton, P. A., and Mellanby, K. The Measurement and Control of Humidity. Bul. Ent. Res. 25: 171-175. 1934.

The grams of KOH used per 100 ml. of water and the specific gravities are presented for solutions maintaining from 10 to 100 percent relative humidity at 30° C.

(8) Stokes, R. H., and Robinson, R. A. Standard Solutions for Humidity Control at 25° C. Jour. Indus. Engin. Chem. 41: 2013. 1949.

The molality of NaOH solutions which will maintain relative humidities from 5 to 95 percent is given at 5-percent intervals.

Reference

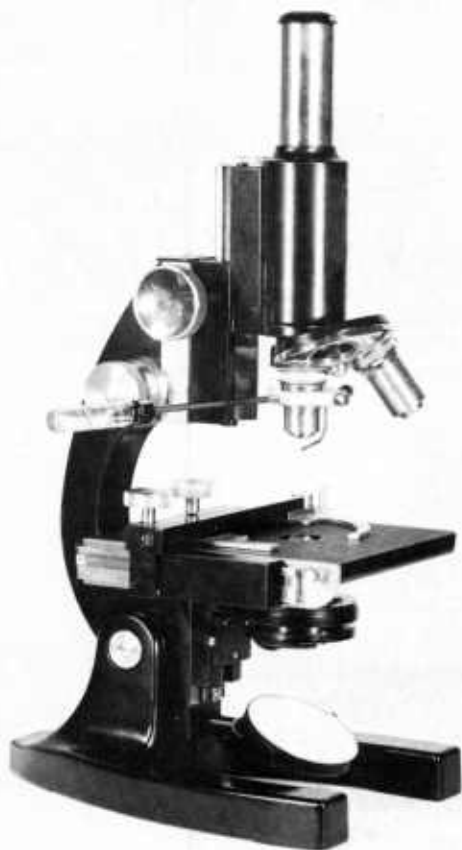
References submitted by R. D. Durbin, Agricultural Research Service, U.S. Department of Agriculture.

Microisolation Device

The isolator illustrated (fig. 55) is of the "biscuit cutter" type and is mounted on the microscope objective so that the cutter remains in the field of view continuously during the isolation of small plant parts, cells, pollen, and spores.

The isolator consists of the following parts: a holder (fig. 56, e) fitted snugly over a 16-mm. microscope objective (d) and held against the base of the objective by a small thumbscrew. Make the holder from a small piece of aluminum or brass approximately 7 mm. thick; fashion a cutting device (b) from a 4-cm. portion of 18-gauge nichrome wire bent as illustrated. Flatten one end of the wire with a hammer and bend the flattened portion into a cylindrical cutter (a) having a diameter of 1.5 mm. Secure the cutter in the holder with a thumbscrew to permit adjustment of its vertical and radial positions. Fix a handle (c) permanently to the holder to facilitate removal for flaming.

Use the isolator as follows: fill convenient containers to a depth of at least 3 mm. with



BN-28937

FIGURE 55.—Microscope with microisolation device attached. (Photo—courtesy of Phytopathology.)

4% clear agar. When agar of less than 4% is used, moisture may be exuded by the pressure of the cutter and the particle being isolated may float out of position. Spread the particles to be isolated individually over the surface of the agar by any of the methods commonly used for spores. Before making isolations, fasten the isolator against the base of the objective with the thumbscrew. Then adjust the cutter in the field of view until it appears slightly above the focal plane. Remove the isolator from the objective and sear the agar surface for a well-placed cell, pollen grain, spore, or whatever particle is involved. If an aseptic transfer is to be made, rack the objective away from the specimen, flame the isolator, and replace it on the objective exactly as before so the cutter automatically lines up in the center of the field of view. Focus the objective until the specimen comes into view through the center of the slightly out-of-focus cutter, then adjust the ob-

jective down until the cutter penetrates the agar to at least twice its depth. On raising the objective, a minute agar plug is removed. Part of this plug with the specimen being isolated on its surface protrudes above the top of the cutter. Remove the protruding agar and specimen for study or transplant. To perform further isolations readily, remove the isolator from the objective, flame the cutter, replace the isolator, and repeat the above procedure.

With this device, the cutter can be observed as it penetrates the agar, the isolator can be removed easily and quickly for flaming, and it can be replaced in exactly the same position without removing the objective from the microscope.

Reference

Leach, C. M. A Simple Device for Single Spore Isolation. *Phytopathology* 45: 405-407. 1955.

Suggestions Concerning the Production of Greenhouse Plants for Experiments With Regulating Chemicals

When experimenting with regulating chemicals, use test plants that are healthy, are of uniform shape and size, and have reached a stage of development most suited to the experiment at hand. To produce test plants efficiently and rapidly, use fresh, well-filled seeds with

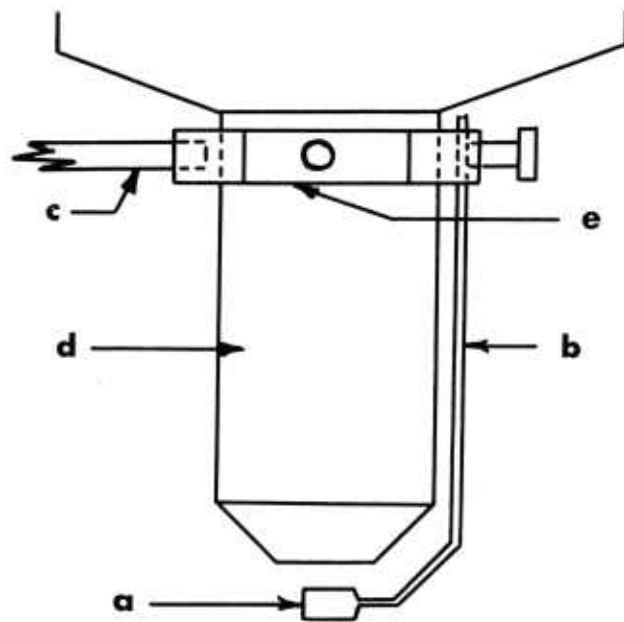


FIGURE 56.—Diagram of isolator. (Illustration—after diagram by C. M. Leach.)

unbroken seed coats, together with the most suitable environmental conditions.

It is, of course, not practical here to describe how all kinds of experimental plants can best be grown. The following shows, therefore, one method of efficiently producing a test plant that has been widely used in experimentation with regulating chemicals, namely, the common bean (*Phaseolus vulgaris* L.).

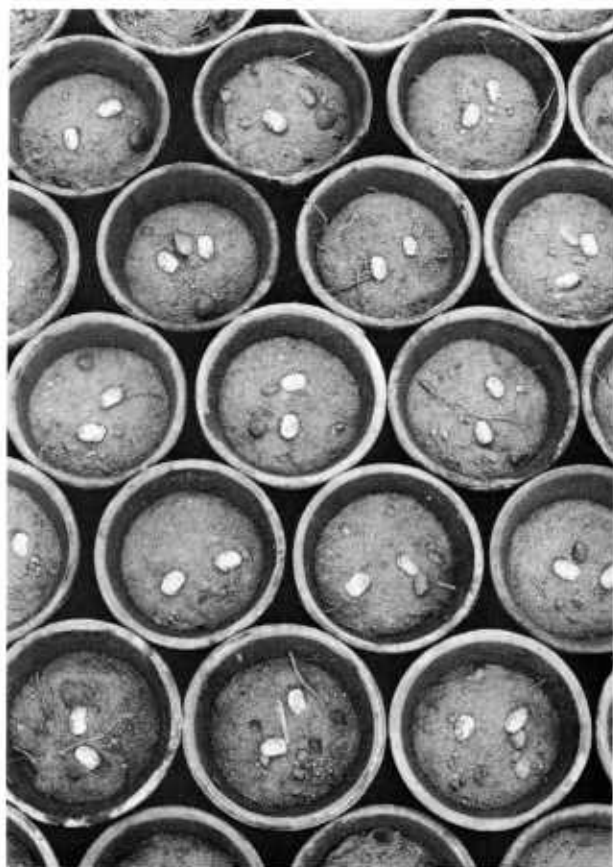
Obtain seeds of a readily available type of bean such as Black Valentine, Pinto, Bountiful, or red kidney types. Use a fertile composted soil that will not pack tightly when placed in a clay pot and watered.

Use small clay pots about 7.5 cm. in diameter. Fill these to within about 2.5 cm. of the rim with the soil. Place from 2–4 bean seeds in each pot, spaced as illustrated in fig. 57. Add enough soil to cover the seeds to a depth of about 1 cm. Use a flat surface to smooth the soil over the seeds but do not pack it. Leave a space of about 1 cm.

from the soil surface to the rim of the pot (fig. 57).

Fill this space with water and allow the water to seep into the soil. Place the filled pots at a temperature of about 27°–32° C. Do not allow the surface of the soil to become even slightly dry.

Young plants will appear above the surface of the soil within 3–4 days. This stage of development is termed the “staple” stage (fig. 58). The amount of light received by plants in the staple stage is critical. To obtain short, sturdy plants, reduce the temperature at this stage to 21°–24° C. and place the plants where they will receive direct sunlight, or expose them to artificial light from fluorescent tubes equal to an intensity of 3,000 foot-candles for 12 hr. each day. To obtain etiolated plants, expose the plants as they break through the soil to a lower light intensity and maintain the temperature at 24°–27° C.



BN-28949

FIGURE 57.—Bean seeds before they are covered with soil.



BN-28946

FIGURE 58.—Bean plants at the “staple” stage of development.

Bean plants grown at the relatively low temperature and high light intensity will develop primary leaves that are approximately 3.5 cm. across within 5–6 days after the seeds are planted (fig. 59). As soon as these leaves are relatively smooth (6–7 days), they are capable of absorbing and translocating regulating chemicals to other parts of the plants. Stems of these plants grow rapidly and respond readily to regulating chemicals that induce cell elongation or cell proliferation, or both.

After the plants have reached a desirable size, sever the stems (just below the surface of the soil) of all but the most suitable plant in each pot. Test plants of uniform size and responsiveness thus can be obtained within 1 wk. or less.

Storage of Seeds Used as a Plant Source for the Study of Hormones and Regulators

To carry out experiments with plant hormones and regulating substances, it is necessary to have seeds of the required kinds that will germinate uniformly and produce vigorous plants. It is also necessary to have a continuous source of a given kind of seed to produce plants that will respond consistently during the experimental period involved. To check biologically any changes in growth-regulating activity that occur in stored hormones and regulating com-

pounds, it is sometimes necessary to use the same lot of seeds over an extended period of time. Conditions most suitable for seed storage are, therefore, essential.

First, obtain the appropriate kind of freshly grown seeds in an amount sufficient to last for a number of years. If the seeds are not uniform in appearance, sort them to eliminate cracked, small, wrinkled, and discolored ones. Make sure, through appropriate tests, that seeds in each lot have a high degree of viability, germinate rapidly, and are relatively free from disease.

To maintain seeds over a long period so they will produce uniform seedlings capable of vigorous growth, store the seeds at a low temperature and with an appropriate moisture content. If seeds are maintained under the conditions listed below, they should remain essentially unchanged for at least 10 years.

Dry the following kinds of seeds to the designated moisture content:

6% moisture

Cucumber
Cocklebur
Radish
Squash
Sunflower
Tobacco
Tomato

8% moisture

Bean
Cereals (including corn)
Pea
Soybean

Do not allow the temperature of the stored seeds to rise above 2° C. If it is more convenient

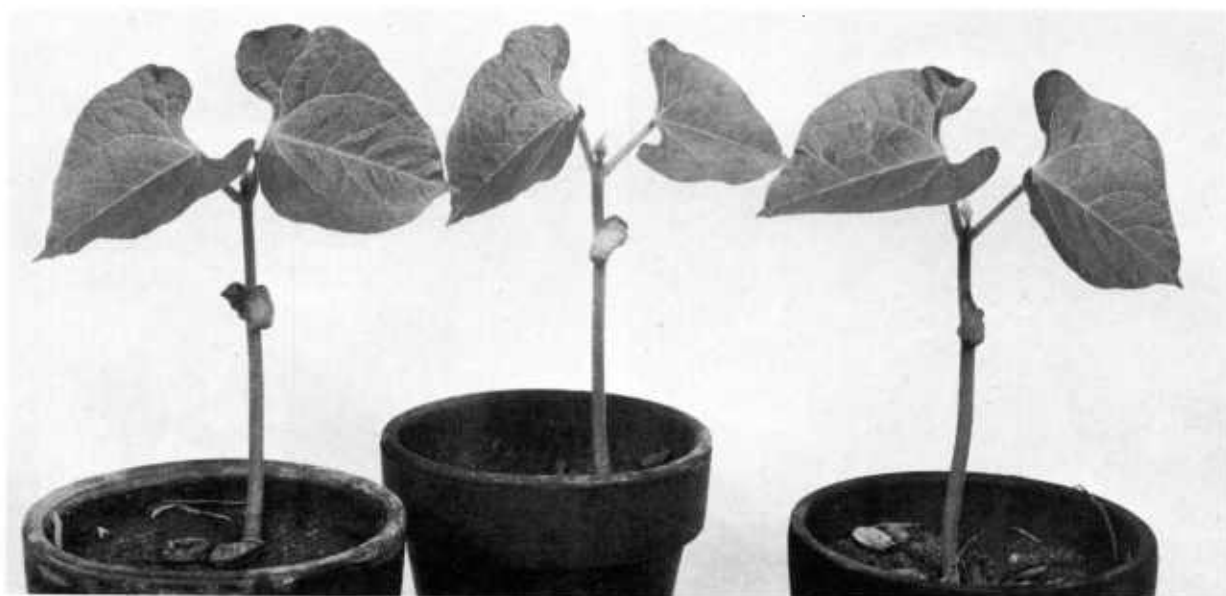


FIGURE 59.—Bean plants 5–6 days after seeds are planted.

to store the seeds in a deep freeze (-10° to -18° C.), moisture content of the seeds may be approximately 1% higher than given above.

To dry the seeds to the desired moisture content, determine the amount of moisture that they contain when they are received. To do this, place 10–20 g. of seeds in a tared dish and weigh accurately. Dry large seeds, such as bean and corn, in a forced-draft oven at 100° – 103° C. for 48 hr. and small seeds for 24 hr. Determine the percentage of moisture lost on the basis of the original fresh weight of the sample.

Spread the entire lot of seeds in a thin layer on a cloth or paper in a warm, dry place with free movement of air, using a heated room or a protected area that receives sunlight. Do not allow the temperature of the seeds to exceed 45° C. After several days, determine the progress of drying by weighing the entire seed lot. From the original weight and from the loss of weight, calculate the moisture content at the time of the weighing as follows:

$$\text{If } a = \frac{\text{desired moisture } \%}{100}$$

and x = weight of water to be lost from lot to reach desired moisture content,

$$\text{then, } \frac{(\text{original weight of water in lot}) - x}{(\text{original weight of lot}) - x} = a$$

$$a(\text{original weight of lot}) - ax = (\text{original weight of water in lot}) - x$$

$$(1-a)x = (\text{original weight of water in lot}) - a(\text{original weight of lot})$$

$$x = \frac{(\text{original weight of water in lot}) - a(\text{original weight of lot})}{1-a}$$

Example:

original weight of seed lot 10,000 g., moisture 16%

1,600 g. water in original lot

$$\text{If } 6\% \text{ moisture is desired, water to be lost } (x) = \frac{1,600 - 0.06(10,000)}{1 - 0.06}$$

$$x = \frac{1,000}{0.94} = 1,063.82 \text{ g.}$$

$$10,000 - 1,063.82 = 8,936.18 \text{ g.} = \text{weight of lot dried to } 6\% \text{ moisture}$$

When the seeds have dried to the desired moisture content, mix the seeds thoroughly and again determine the moisture content with a final test.

In order to facilitate removal of small amounts of seeds from storage for a series of tests without raising the temperature and without changing the moisture content of the remaining seeds, divide the entire seed lot into small samples, each sample adequate for use over a short period. Place each sample in a separate cellophane or polyethylene sack with a label describing the seed lot. Place the sacks into Mason jars and seal them. Store the jars at the appropriate temperature. When seeds are needed, remove a sack quickly and reseal the jar to avoid increasing the temperature or moisture of the remaining seeds.

Reference

Information assembled by E. H. Toole, Agricultural Research Service, U.S. Department of Agriculture.

OBSERVING AND MEASURING PLANTS

Aphid Honeydew Technique for Study of Sieve Tube Content

When aphids are allowed to feed on the stems of rooted willow cuttings, the leaves of which have been supplied with $C^{14}O_2$, honeydew from the insects contains radioactive sugar. The fact that aphids feed within sieve tubes offers a means of detecting and, under certain conditions, measuring the tagged sugar in these tubes.

The honeydew method is described here on the assumption that some radioactively tagged growth-regulating substances, when carried in the sieve tubes, might be ingested by the aphids and then excreted along with honeydew, thus offering a means of determining whether the radioactivity reached a known position in the stem by moving through the sieve tubes. This method is not suitable, however, for studying the metabolism of transported regulating sub-

stances since the tagged molecules must pass through an insect which might in itself metabolize the compound.

Use the aphid *Tuberolachnus salignus* (Gmelin) (Homoptera, Aphididae). Cut segments about 15 cm. long from branches of 2- to 4-yr.-old willow trees, *Salix viminalis* L. Immerse the lower 4-5 cm. of each cutting in water and, when the new roots are 3-4 cm. long, pot the cuttings in a mixture of peat moss and sand. Supply the plants with natural light or with artificial light from a suitable source that gives an intensity of at least 500-1,000 foot-candles at the leaf surfaces.

Apply a C^{14} -tagged regulator to the leaves. (See "Bean stem curvature method (absorption and translocation)," p. 12, fig. 7, for application.) Collect the honeydew from the aphids feeding on stems of the cuttings near leaves to which the regulator has been applied (G. P. Hill, The Sources of Sugars in Sieve-Tube Sap. Ann. Bot. (n.s.) 27: 79-87. 1963). Place the honeydew directly on a smooth nickel planchet. Flood the planchet with distilled water so the honeydew is dissolved and spread evenly over the entire surface of the planchet. Evaporate the water at room temperature and then bring the planchets to constant weight in an oven at 22° C. Finally, dry the planchets for several days in a desiccator. Count the samples with a gas-flow counter and calculate the results as counts/minute/mg. of sample. Self-absorption would, of course, be involved in the use of this method. The amount of self-absorption could be estimated by mixing known amounts of the radioactively tagged regulator with weighed amounts of honeydew collected from aphids feeding on cuttings to which the regulating substance has not been applied.

Reference

Based on research by A. J. Peel and P. E. Weatherley. Studies in Sieve-Tube Exudation Through Aphid Mouth-Parts: The Effects of Light and Girdling. Ann. Bot. (n.s.) 26: 633-646. 1962.

Aphid Stylet Technique for Studying Regulating Substances in Sieve Tubes

Aphid mouth parts have been used to study the content of sieve tubes and of single sieve elements. This technique is described here with the thought that it might also be useful for measuring radioactively tagged regulating substances and metabolites of them translocated in sieve tubes.

Anesthetize aphids, *Tuberolachnus salignus* (Gmelin) (Homoptera, Aphididae), with a gentle stream of CO_2 while they are feeding on

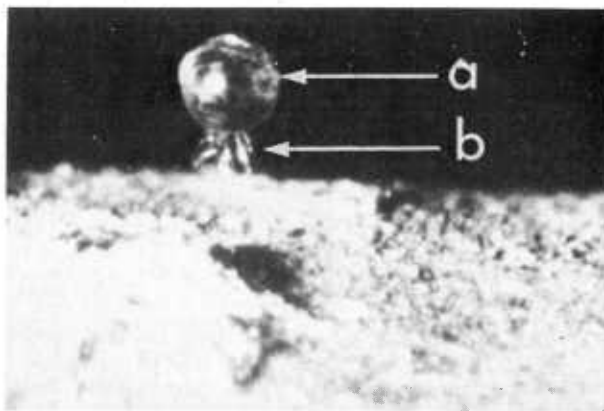
stems of willow cuttings, the leaves of which have been treated with a radioactive growth regulator. (See "Bean stem curvature method (absorption and translocation)," p. 12, fig. 7, for application.) Using a binocular microscope and a fine scalpel made from a sliver of a razor blade, sever the proboscis of one of the insects as close to the head as possible. Remove the labial sheath surrounding the stylet with a fine brush. Repeat the procedure, severing as many embedded stylets as are needed to obtain a sufficient amount of exudate (fig. 60). Collect the sieve tube exudate using a glass capillary (0.5-0.1 mm. inside diameter) held with a manipulator against the surface of the exuded droplet. Measure the length of the column of liquid in the capillary with a vernier microscope and calculate its volume. Exudates collected in this way can be subjected to various chromatographic techniques to separate out the tagged growth regulator or metabolites of it. Measure the tagged regulating substance present by conventional counting methods or by radioautography.

Reference

Based on research by P. E. Weatherley, A. J. Peel, and G. P. Hill. The Physiology of the Sieve Tube. Preliminary Experiments Using Aphid Mouth Parts. Jour. Expt. Bot. 10: 1-16. 1959.

Extraction of Hormones and Growth-Regulating Substances From Plants

Endogenous hormones can be obtained by allowing these substances to diffuse from freshly cut plant surfaces into agar. A method useful for this purpose is described on page 96.



BN-28954

FIGURE 60.—Phloem exudate (a) that collected on detached aphid stylet (b) embedded in a stem. (Photo—courtesy of M. H. Zimmermann and Science.)

Methods of chemically extracting hormones and growth-regulating substances from plants are well known and widely used in physiological studies. Since some of these methods were recently described by Mitchell and Smale (cited below), a detailed description of them is not given here.

The amount of extractable hormone in a plant or plant part may vary, depending upon environmental conditions and factors controlling hormone synthesis, utilization, and degradation. Solvent used, pH, and also some physical factors can influence the amount of hormones obtainable from a plant part. For example, the amount of extractable hormone in some kinds of stems has been increased by subjecting the plant part to a centrifugal force (Kawase, Monselise). It is not known whether this increase is due to increased hormone synthesis, to an unbinding effect of centrifugation that accelerates outward movement of the hormone, or to accelerated basipetal transport of hormones that makes them readily extractable. Regardless of the mechanism involved, the investigator should bear in mind that quantitative extraction of plant hor-

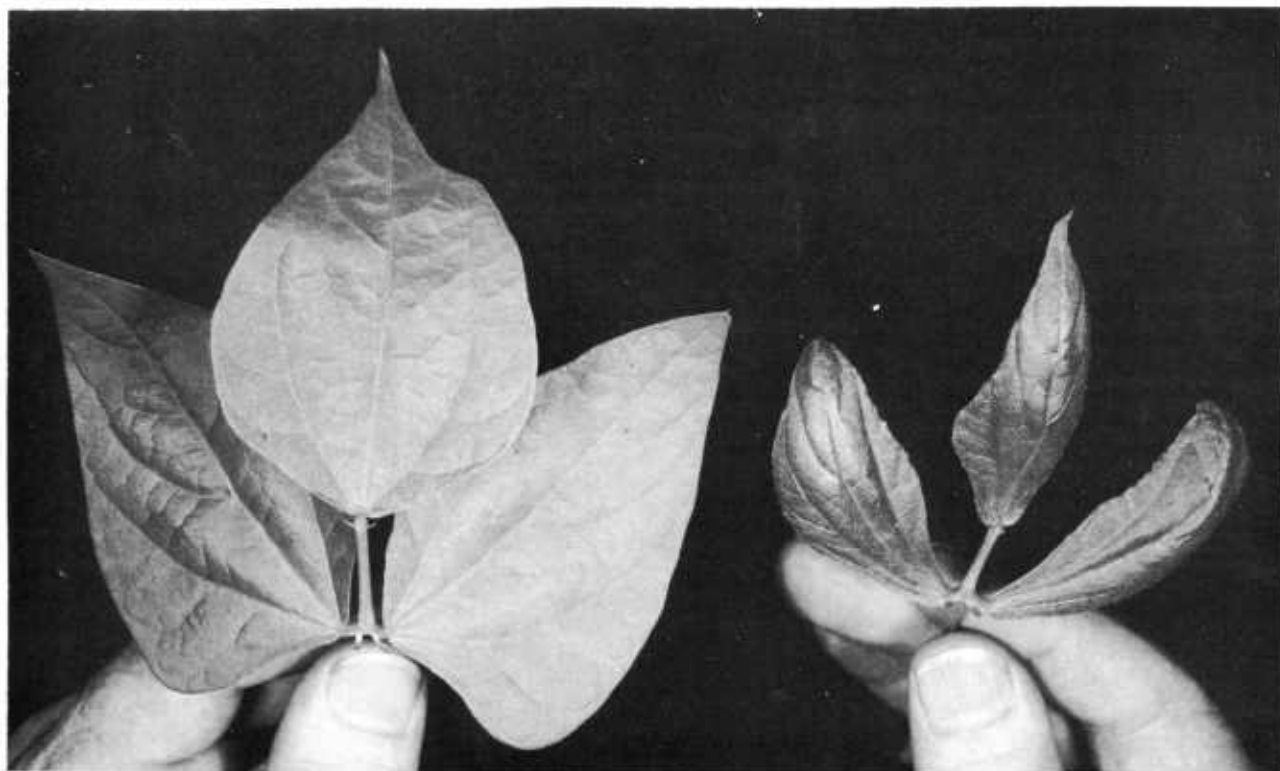
mones, using any of the present methods, depends on several factors and that the results may not be truly quantitative.

References

- Goldschmidt, E. E., and S. P. Monselise. Private communication. Unpublished data. 1966.
 Kawase, Makoto. Centrifugation, Rhizocaline and Rooting in *Salix alba* L. *Physiol. Plant.* 17: 855-865. 1964.
 Mitchell, J. W., and Smale, B. C. Bioassay—Plants. *Analytical Methods for Pesticides and Food Additives*. v. 1, pp. 176-191. Academic Press, Inc., New York. 1963.

Formative Effects of Regulating Chemicals

Some regulating compounds cause plants to change their usual pattern of growth, especially their pattern of leaf growth. Very minute amounts of such compounds as 2,4-dichlorophenoxyacetic acid on such plants as cotton, bean, and grape often result in relatively small leaves with narrow curled blades and sometimes enlarged veins (fig. 61, right).



PN-305

FIGURE 61.—Right, Malformed leaf from a plant treated with a small amount of growth-regulating substance, compared with (left) a comparable leaf from an untreated plant.

Interferometer Method of Detecting Minute Growth Movements

Minute plant movements associated with growth response induced with hormones and regulators can be detected by means of a sensitive interferometer method. Support a microscope slide (fig. 62, *b*) by placing one end in a groove in a wooden block. Glue 2 sharp glass pegs to one end of a shorter slide. Rest one slide against the other with the shorter one supported by the pegs. Using cocoa butter, fasten one end of a fine glass rod to the shorter slide, the other end to the leaf or other part of a plant where movement is to be detected. Heat sodium chloride in a porcelain dish or spoon (*c*) over a flame so yellow light made by the sodium chloride reflects from the surfaces of the slides. Focus a horizontal telescope (*a*) equipped with cross hairs on the image of the flame reflected from the glass. Observe the interference rings that are apparent when the inner surfaces of the slides are the appropriate distance apart. Without producing air currents, allow small spray droplets of the test solution (with a wetting agent added, if needed) to strike the leaf or other portion of the plant used. Movement

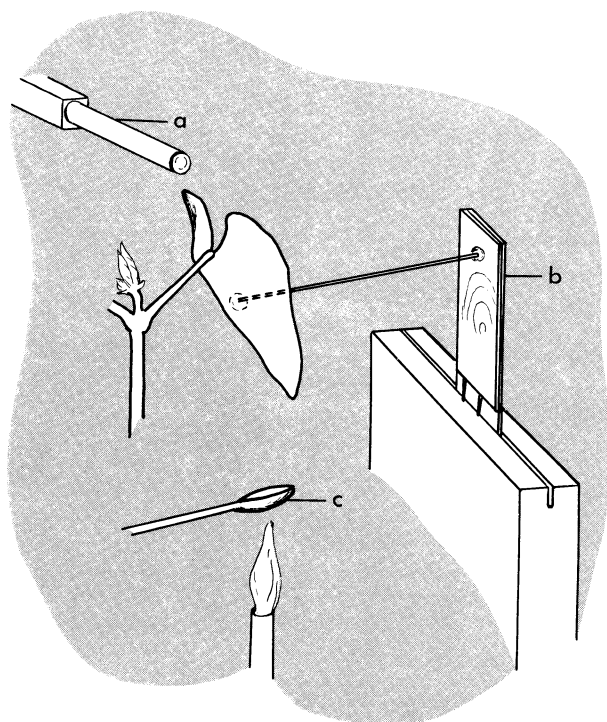


FIGURE 62.—Diagram of apparatus used to detect minute growth movements.

of the interference rings in one direction indicates movement of the plant, the distances involved being in the range of wavelengths of the light used.

Freehand Sections for Studying Cell Responses to Regulators

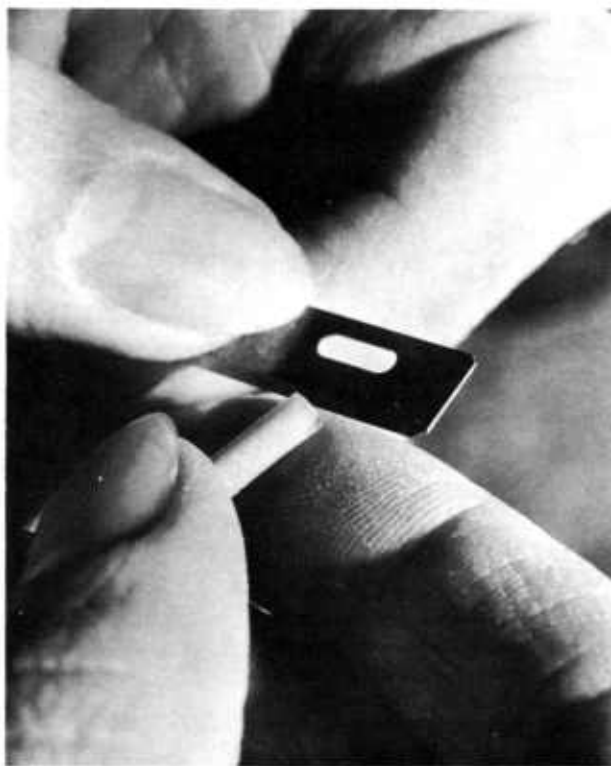
In studying cellular responses to hormones and regulating substances, it is sometimes desirable, or even essential, that living cells be used so that the actual size, shape, and behavior of the cell parts can be observed. Freehand sections can be made of succulent parts of plants that contain uninjured cells suitable for detailed studies of mitochondria and larger cell parts and for use in still and cinema photography in recording the responses to hormones and regulators.

To make freehand sections of succulent tissue, use the sharpest single-edge razor blades possible and discard each blade at the first evidence that it has become dull. In the case of new blades, without touching the sharp edge, remove excess oil or grease with tissue. Dip the blade several times in acetone to remove any remaining oil. Hold the segment to be sectioned under a fluorescent lamp with magnifying glass attached.

The plane of the blade should pass through the cells parallel with the long axis of the plant segment, i.e., parallel with the long axis of the cells (fig. 63). If the plane of the blade is not parallel with the long axis of the segment, the number of cells injured is greatly increased, probably due to development of greater stress in the tissue. Make the sections about 35–40 microns thick. After each slice, make sure the blade is held in a position that maintains the plane of the cut surface parallel with the long axis of the segment.

Move the blade through the tissue so the cutting edge forms an angle 15° off perpendicular with the long axis of the segment. Slide the blade through the tissue at a uniform rate. Avoid a sawing motion. Keep the blade and the tissue wet during the entire procedure and immediately transfer the tissue section to tapwater that has been boiled to remove any chlorine and then aerated to replace the dissolved air driven off during the boiling. This tapwater should be at room temperature. In making the transfer, float the section off the razor blade by immersing the blade in the water.

To observe the tissue microscopically, transfer each section from the water in which it has been stored temporarily by floating it into a droplet of the water in the well of a special slide



BN-28948

FIGURE 63.—Making freehand sections for studying cell responses to regulators.

chamber. (See "Slide chamber for the study of living cells," p. 123.)

In the microscopic study of the living cells, it is generally necessary to ignore the first row below the cut surface since these are usually injured in the process of making the section. The second row below the cut surface may contain injured cells, but all or most of the cells in the third row are generally uninjured.

Reference

Submitted by Marjorie D. Montgillion, Agricultural Research Service, U.S. Department of Agriculture.

Measurement of Root Response

Place a disk of blotting paper impregnated with a water mixture of the regulating chemical and another impregnated with tapwater or distilled water in separate petri dishes. Ordinary blotting papers often contain chemicals that inhibit root growth. Blotting paper especially prepared for seed tests can be obtained

from the Standard Paper Manufacturing Co., P.O. Box 1554, Richmond, Va. 23212.

Place several cucumber seeds of uniform size on the surface of each paper. Store the closed dishes at room temperature in darkness for 24 hr.; then measure growth of the roots repeatedly during the following 2 or 3 days with the dishes closed (fig. 64). Compare growth of the roots on the chemically treated papers with that of roots on the papers impregnated with water.

Radioautographs of Sectioned Plant Material

Although problems still remain to be solved, radioautographs of sectioned plant material have been obtained that show clearly the position of the tagged compounds within various tissues. Attempts to use radioautographs to locate soluble tagged compounds within the cells, however, have not been very successful. The following procedures are described in general with the hope that they will be useful in locating tagged regulators in tissues at least, and perhaps, with some modification, certain of these will be useful for intracellular studies. Conventional methods cannot always be used to fix, embed, and section the tissues to prepare them for radioautography because many growth-regulating substances are soluble in the alcohol and in some of the other liquids used in these procedures.

One method for tissue radioautographs involves cutting the sections while the tissue is frozen and then exposing the sections to an appropriate type of film at a temperature below freezing. A cryostat is the most suitable apparatus for this purpose since, with it, sections can be made easily at very low temperatures.

Wrap aluminum foil around a pencil to make a cylinder about 1 cm. long. Fill the metallic cylinder with liquid gelatin (10%) and place a small piece of the plant tissue in the gelatin. Then freeze the gelatin and tissue in the cryostat at -16° to -20° C. Place a drop of water on the microtome specimen holder and freeze the gelatin block in place for sectioning. While operating the cryostat, cut the desired number of sections 10–25 microns thick.

Within the cryostat, using a red safe light (Wratten Series No. 1), place the sections flat against the emulsion of a Kodak Type A lantern slide. X-ray film cut into a suitable size may be substituted for the lantern slide. Place a freshly and carefully cleaned microscope slide or one freshly coated with silicon above the sections and clamp the 2 slides together with binder clips. Place this assembly in a light-tight container and expose the emulsion



PN-302

FIGURE 64.—Measuring root responses.

to the radioactivity within the cryostat or transfer without thawing to a freezer compartment. Prepare several slides this way so they can be developed one at a time at various intervals to determine the correct exposure. In making this test, the exposure time must be doubled in order to increase the density of the image significantly. In developing the emulsion, use methods suggested by the manufacturer of the particular emulsion. For example, if the Type A lantern slide is used, develop in D-19 developer for 4 minutes, stop in a bath of water for 15 seconds, fix in 20% hypo for 20 minutes, and then wash thoroughly in water. The sections of plant tissue that survive the development and fixation are in place, and thus the position of radioactivity in relation to different tissues and cells can be determined.

Reference

Procedure compiled by J. F. Worley, Agricultural Research Service, U.S. Department of Agriculture.

Two other types of sensitive emulsions have been used, namely liquid emulsion and stripping film. To use these emulsions, the tissue must be fixed and then embedded or dried. It is emphasized that in the process of fixing, embedding, or drying, soluble substances may be removed from the tissue. In addition, the position of the substance in question or the position of the cell part with which it is associated may change during various steps in the procedure. Some references concerning use of these emulsions are:

- Belanger, L. F., and Leblond, C. P. A Method for Locating Radioactive Elements in Tissues by Covering Histological Sections With a Photographic Emulsion. *Endocrinology* 39: 8-13. 1946.
- Fitzgerald, P. J., Simmel, Eva, Weinstein, Jerry, and Martin, Cynthia. *Radioautography: Theory, Technic, and Applications*. Lab. Invest. 2: 181-222. 1953.
- Kamen, M. D. *Radioactive Tracers in Biology*, An Introduction to Tracer Methodology. Academic Press, New York. Ed. 2, 429 pp. 1951.
- Pelc, S. R. Autoradiograph Technique. *Nature* 160: 749-750. 1947.

Separation of Leaf Areas Treated With a Regulating Chemical From the Remaining Untreated Portions of the Leaf

In studying the movement of radioactively tagged regulating chemicals in leaves, it is often desirable to remove the treated portion of a leaf so that the untreated portion which remains can be assayed for radioactivity. The following simplified method is useful since the equipment required is inexpensive and, if accidentally contaminated, can be discarded, thus minimizing the danger of contaminating untreated portions of the leaf. Place the detached leaf on a piece of sandpaper (C weight, 3/0-120) or emery cloth. Place the open end of a vial over the treated portion of the leaf so the inside edge of the vial does not come in contact with the radioactive material on the leaf. Turn the vial back and forth with slight pressure and thus cut out and remove the treated area

of the leaf (fig. 65). Treated portions of several leaves can be removed without contaminating untreated portions by using only 1 vial and 1 piece of abrasive material and then discarding the equipment.

Slide Chamber for the Study of Living Cells

Place a No. 1 square cover glass on top of a No. 2 square cover glass and align their edges accurately. Use paraffin melted with a hot needle to seal the edges (fig. 66, y , y') of 2 pairs of aligned cover glasses (x , x') to opposite ends of a microscope slide. Immerse the sections of plant tissue in tapwater placed in the center of the well formed by the square cover glasses. Cover the sections with rectangular cover glass (a) supported as a bridge about 0.26-0.32 mm. above the glass slide by the square cover glasses. Use a slide 25 mm. wide

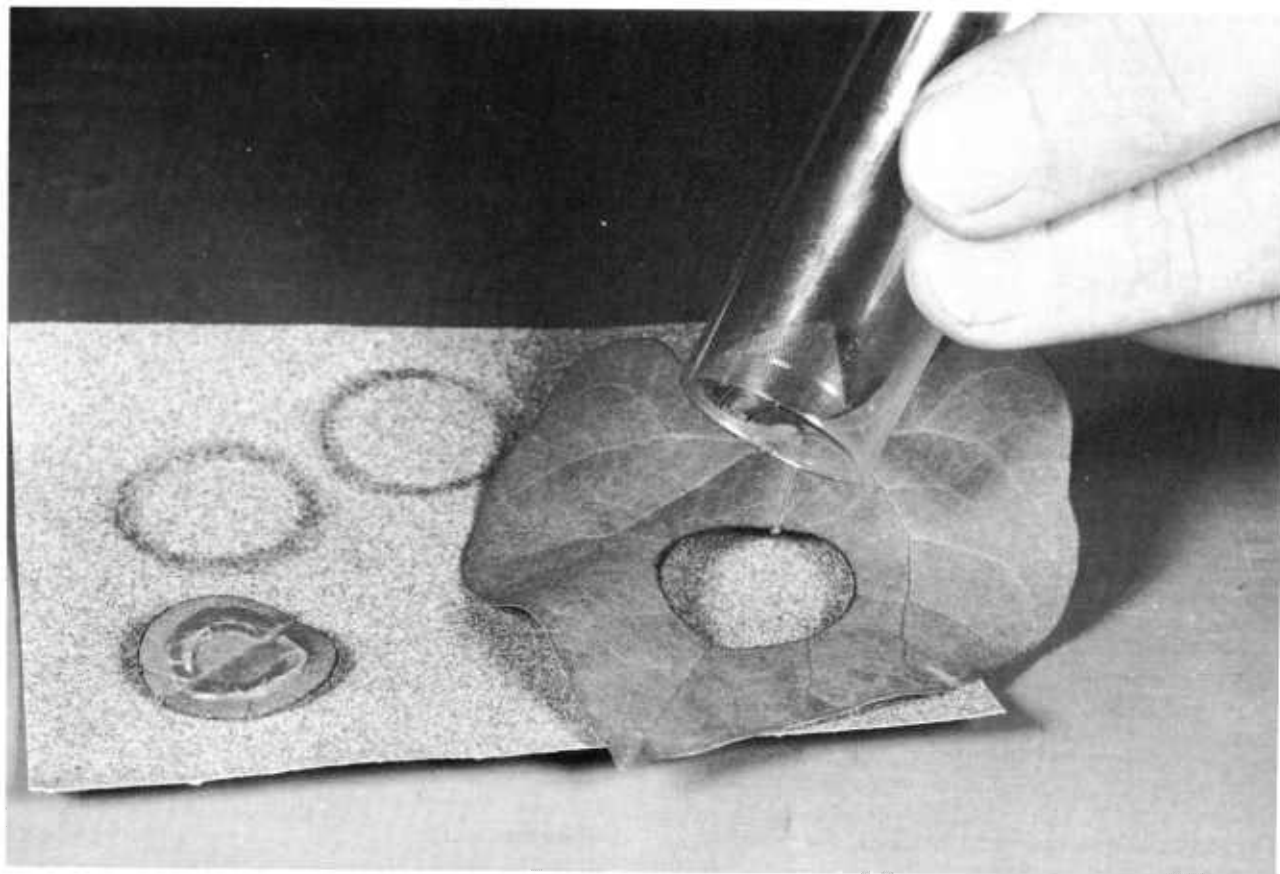


FIGURE 65.—Removing the treated area of a leaf.

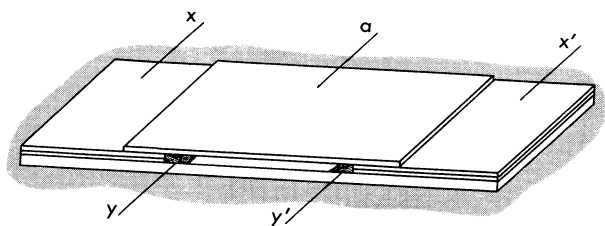


FIGURE 66.—Diagram of slide chamber for studying living cells.

(narrow dimension), square cover glasses 25 mm. wide, and a rectangular cover glass 22 mm. wide.

Terminal Growth Measurements

Many regulating chemicals cause an increase or a decrease in the rate of growth of the main stems of plants. The effect of a compound on the length of the stems is therefore one means of evaluating the regulating properties of the chemical.

Apply the chemical in a suitable carrier (lanolin or aqueous mixture), for example, to bean plants that have developed primary leaves that are partially expanded (3.5–5 cm. across) and when the trifoliolate leaves are still folded in the terminal bud (fig. 67, *left*). Measure the length of the terminal bud from second node to tip of bud at the time of treatment. Measure from the same node (second) to the tip of the terminal bud about 1 wk. after the treatment (fig. 67, *right*), and compare growth with comparable measurements of untreated plants.

Observation Chamber for Continuous Application of Hormones or Growth Regulators to Living Cells

Obtain an observation chamber^s (fig. 68, *a*). Prepare sections of living tissues to be studied as described on p. 120. Place a glass plate (*b*), made by cutting a microscope slide to the required size, on the coverslip that serves as the lower side of the chamber. Place into position the soft plastic washer that separates the two glass sides that form the top and bottom of the chamber. Fill the chamber with water or the test solution, immerse the freshly cut sections in the liquid, and position them directly over the glass plate. Close the chamber by dropping the second coverslip into position on the plastic

washer. Do this carefully so air bubbles are not trapped within the chamber. Apply the metal washer and threaded ring. Tighten the threaded ring to press the coverslips gently against the plastic washer. Construct inlet and outlet tubes with 25-gauge hypodermic needles and insert these through the plastic washer as illustrated (*c*). Connect the chamber to an electrolytic pump (*d*) or to a constant-flow mechanical pump if relatively large amounts of liquid are used. Observe or record photographically cellular responses by placing the assembly under a microscope.

Motion Detector for Recording Plant Growth

This sensitive instrument (fig. 69) can be made to detect, measure, and record growth and minute movements of plants continuously, including even slight growth responses to hormones and regulating chemicals.

The basic system consists of an angle transducer connected to the plant with a counterbalanced variable-length rod. Output from the transducer is adjustable by means of a voltage divider so that the voltage matches the desired sensitivity of a recorder.

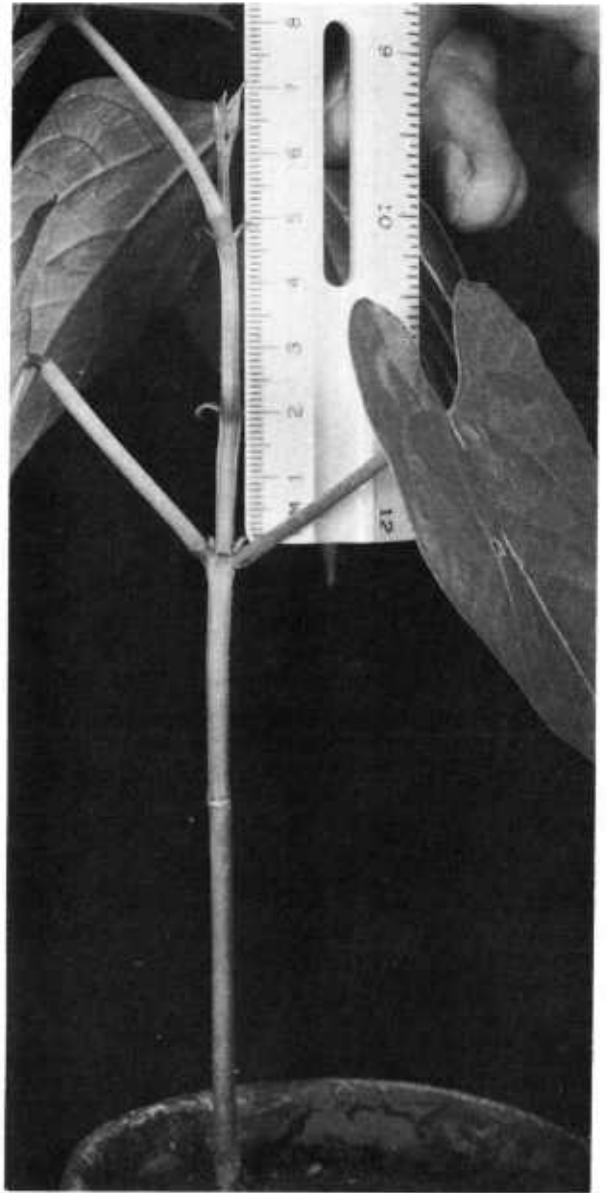
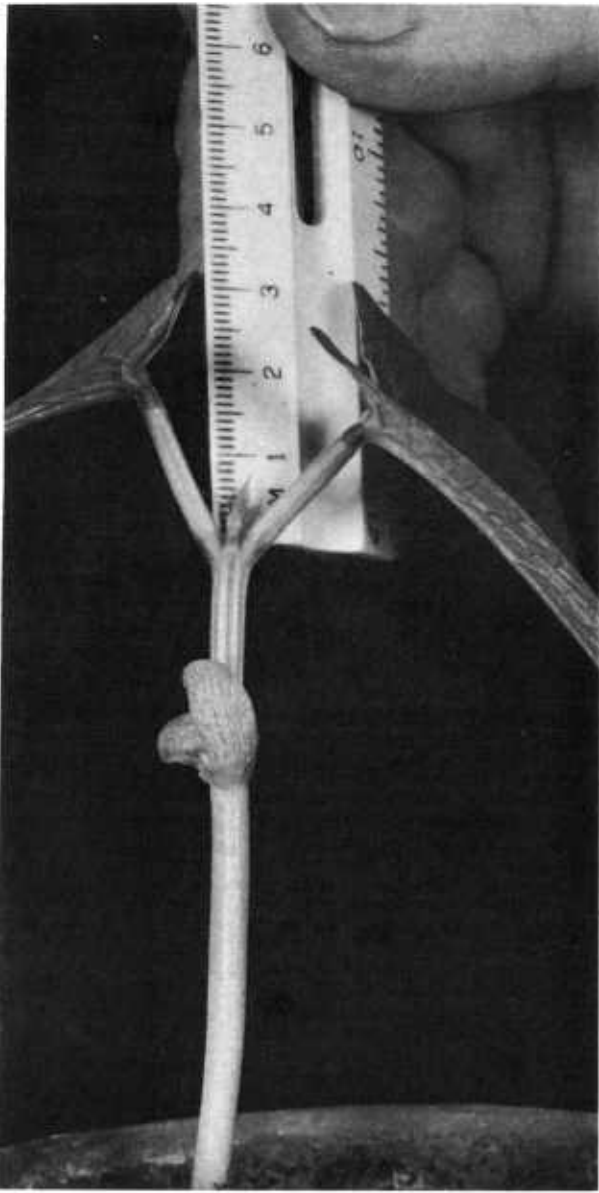
Obtain an angle transducer with a d.c. voltage output in excess of the recorder. Connect the transducer to a voltage divider and then connect the divider to a recorder such as a 0–10 mv data logger. Attach a part of an aluminum, rifle-cleaning rod to the transducer shaft so that the rod is counterbalanced and free to move vertically (fig. 70). Adjust the length of the rod by adding sections when needed.

Construct a spring clip that can be attached to the stem or leaf of the plant to be studied and fasten this clip to the counterbalanced rod. Adjust the counterbalance so that the tip has a tracking weight of less than 0.25 g. Connect the transducer to the voltage divider and recorder. Adjust the voltage divider and rod length so that the sensitivity of the instrument (as fine as 5×10^{-4} in.) meets your needs.

To make an arrangement with which both vertical and horizontal movements of plants can be measured, mount the sensor portion of a second transducer piggyback fashion and at a 90° angle in relation to the first one (fig. 70). Arrange the first transducer to measure the vertical movement and the second to measure the horizontal movement in a plane perpendicular to the connecting rod.

To prevent the recorder from moving off scale, place the transducer system on a motor-

^s Obtainable from the Baltimore Instrument Co., Inc., 716 West Redwood Street, Baltimore, Md. 21201.



PN-308, A and B

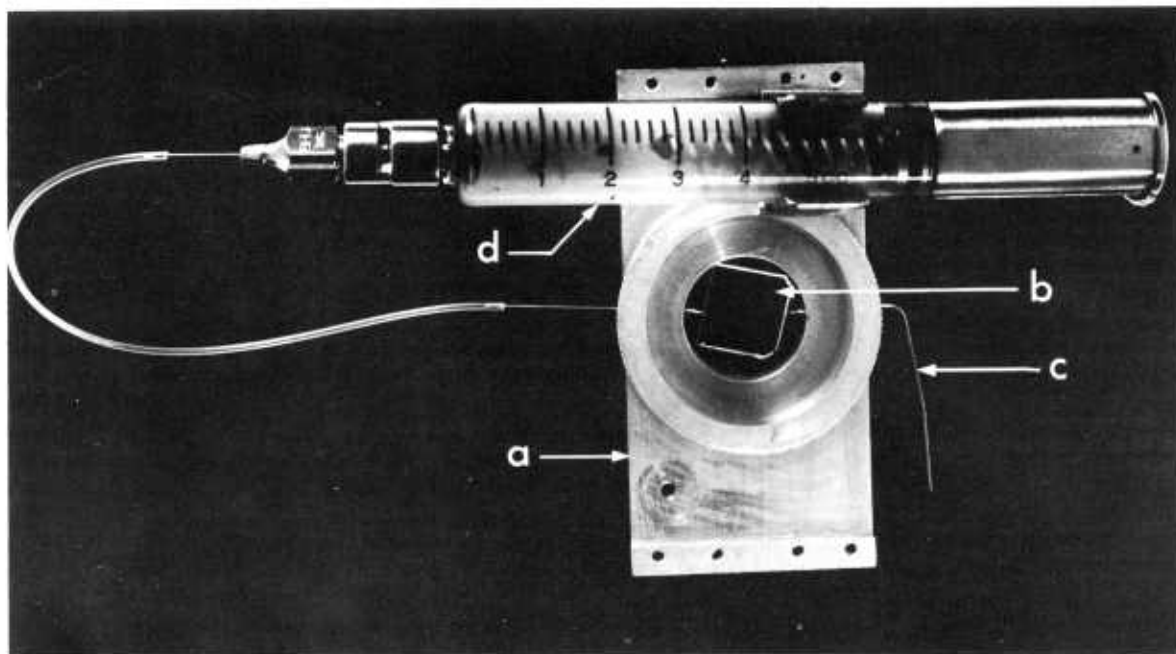
FIGURE 67.—Measuring terminal growth.

ized jack and control the height of the jack platform by a feedback system for the output of the transducer. Arrange the system (fig. 71) so that when the output of the transducer reaches a preset upper limit, the motor that operates the jack is energized, thus raising the platform upon which the transducer system rests. The transducer output then decreases until it

reaches a lower preset mark and the motor is stopped. Adjust the upper and lower limits of recorder movement by variable potentiometers.

Reference

Method developed by H. H. Kleuter, R. J. Downs, W. A. Bailey, and D. T. Krizek, Agricultural Research Service, U.S. Department of Agriculture.



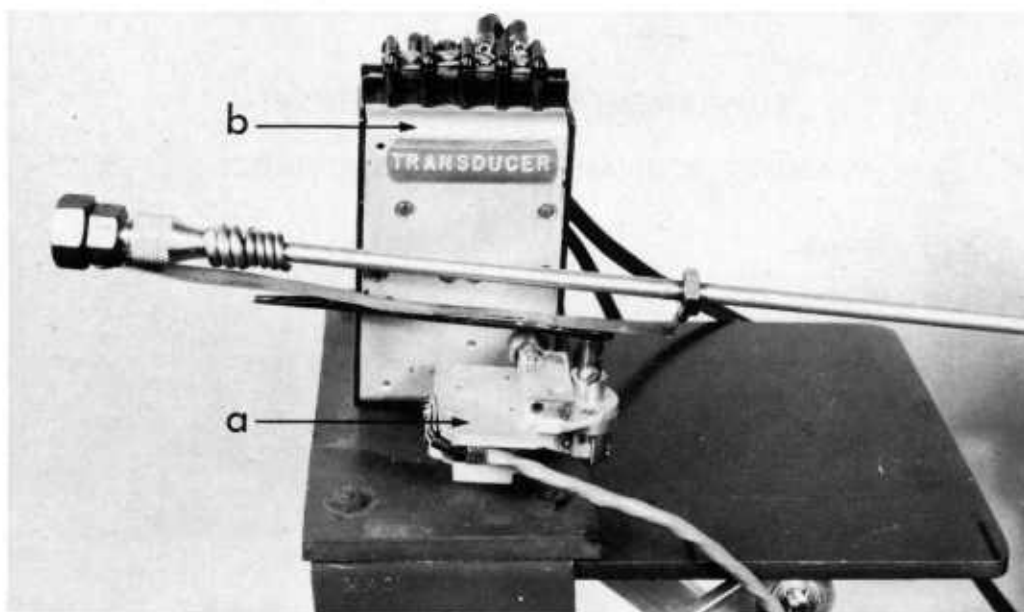
BN-28945

FIGURE 68.—Observation chamber for continuous application of hormones or growth regulators to living cells: (a) Plate of observation chamber; (b) glass plate; (c) hypodermic needle that serves as outlet; (d) electrolytic pump.



BN-28944

FIGURE 69.—Complete motion detector with counterbalanced rod attached to plant.



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FIGURE 70.—Method of counterbalancing rod that conveys plant movement to transducer. Sensor portion of a transducer (*a*) bearing the counterbalanced variable-length rod is mounted piggyback at a right angle to the intact transducer (*b*).

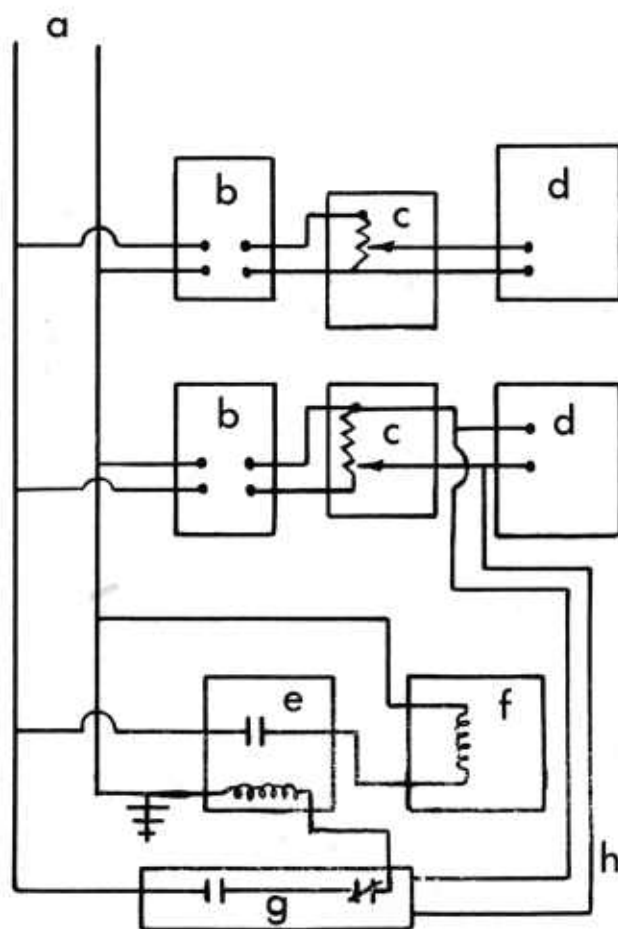


FIGURE 71.—Schematic drawing showing method of wiring apparatus when arranged to prevent the recorder from moving off scale: *a*, 110 volt a.c. current supply; *b*, transducer; *c*, voltage divider; *d*, recorder; *e*, relay; *f*, motor connected with jack; *g*, high-low limit control switch; *h*, feedback lines.

SUPPLEMENTARY INFORMATION

MEASURES, EQUIVALENTS, AND ABBREVIATIONS

WEIGHTS

- 1 kilogram (kg.) = 1,000 grams (g.) = 2.2 pounds (lb.)
- 1 gram (g.) = 1,000 milligrams (mg.) = .035 ounce avoirdupois (oz.)
- 1 milligram (mg.) = 1,000 micrograms (γ or gamma)
- 1 pound (lb.) = 16 ounces avoirdupois = 453.6 grams or 0.45 kilogram
- 1 ounce avoirdupois (oz.) = 28.35 grams
- 1 pint (pt.) of water weighs approximately 1 pound
- 1 gallon (gal.) of water weighs approximately 8.34 pounds

VOLUMES

- 1 liter (l.) = 1,000 milliliters (ml.) = 1.057 fluid quarts (qt.)
- 1 milliliter (ml.) or cubic centimeter (cc.) = 0.034 fluid ounce
- 1 milliliter or cubic centimeter of water weighs 1 gram
- 1 liter of water weighs 1 kilogram
- 1 gram in 1 liter = 1,000 parts per million (p.p.m.) or 0.1%
- 1 gallon (gal.) = 4 quarts = 3.785 liters
- 1 quart (qt.) = 2 pints = 0.946 liter
- 1 pint (pt.) = 16 fluid ounces = 0.473 liter
- 1 fluid ounce (oz.) = 29.6 milliliters or cubic centimeters
- 1 gallon (gal.) = 16 cups = 128 fluid ounces
- 1 quart (qt.) = 4 cups = 32 fluid ounces = 64 tablespoons
- 1 pint (pt.) = 2 cups = 16 fluid ounces = 32 tablespoons
- 1 cup = 8 fluid ounces = 16 tablespoons = 48 teaspoons

- 1 fluid ounce (oz.) = 2 tablespoons = 6 teaspoons
- 1 tablespoon (tbsp.) = 3 teaspoons (tsp.)

LENGTHS

- 1 mile (mi.) = 5,280 feet = 1.609 kilometers
- 1 rod (rd) = 16.5 feet = 5.029 meters
- 1 yard (yd.) = 3 feet = 0.914 meter
- 1 foot (ft.) = 12 inches = 0.305 meter
- 1 inch (in.) = 2.540 centimeters
- 1 kilometer (km.) = 1,000 meters = 0.621 mile
- 1 meter (m.) = 100 centimeters = 3.281 feet
- 1 centimeter (cm.) = 10 millimeters = 0.394 inch
- 1 millimeter (mm.) = 0.039 inch = 1,000 microns
- 1 micron (μ .) = 1,000 millimicrons = 0.0000394 inch
- 1 millimicron (m μ .) = 10 angstrom units
- 1 square kilometer (sq. km.) = 1,000,000 square meters = 0.386 square mile
- 1 square meter (sq. m.) = 10,000 square centimeters = 10.764 square feet
- 1 square centimeter (sq. cm.) = 100 square millimeters = 0.155 square inch
- 1 square millimeter (sq. mm.) = 0.002 square inch

AREAS

- 1 square mile (sq. mi.) = 640 acres = 2.590 square kilometers
- 1 acre (A.) = 160 square rods = 4046.873 square meters = 43,560 square feet
- 1 square rod (sq. rd.) = 30.25 square yards = 25.293 square meters
- 1 square yard (sq. yd.) = 9 square feet = 0.836 square meter
- 1 square foot (sq. ft.) = 144 square inches = 0.93 square meter
- 1 square inch (sq. in.) = 6.452 square centimeters

TEMPERATURE CONVERSIONS

Short-Cut Method of Converting From Centigrade to Fahrenheit Degrees

Double the Centigrade reading, subtract 10% of this value, and add 32.⁹

Conventional Method of Converting From Centigrade to Fahrenheit Degrees

Multiply the Centigrade reading by nine-fifths and add 32.

$$(^{\circ}\text{C.} \times 9/5) + 32 = ^{\circ}\text{F.}$$

Conventional Method of Converting from Fahrenheit to Centigrade Degrees

Subtract 32 from the Fahrenheit reading and multiply by five-ninths.

$$(^{\circ}\text{F.} - 32) \times 5/9 = ^{\circ}\text{C.}$$

⁹ Reprinted by permission from Chemical and Engineering News, Nov. 21, 1949.

METHODS OF MAKING NUTRIENT SOLUTIONS

If plants are to be grown only for 2–3 wk. in nutrient solution, a suitable nutrient can be prepared by adding only macronutrients to tapwater. If the plants are to be grown for a longer period, however, or if distilled water is used, micronutrients must generally be added.

Nutrient Solution Made With Tapwater

Prepare a $\frac{1}{2}$ -molar stock solution of each of the following macronutrients in separate containers using tapwater:

KH_2PO_4 (68.07 g. per liter)
 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (123.25 g. per liter)
 $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ (109.54 g. per liter)
 NaNO_3 (42.50 g. per liter)

To make the nutrient solution, add the macronutrients from the stock solutions in the following amounts to 50 liters of tapwater:

156 ml. of $\frac{1}{2}$ -molar solution of KH_2PO_4
 581 ml. of $\frac{1}{2}$ -molar solution of MgSO_4
 $\cdot 7\text{H}_2\text{O}$
 306 ml. of $\frac{1}{2}$ -molar solution of CaCl_2
 $\cdot 6\text{H}_2\text{O}$
 375 ml. of $\frac{1}{2}$ -molar solution of NaNO_3

Nutrient Solution Made With Distilled Water

Prepare a $\frac{1}{2}$ -molar stock solution of each of the following macronutrients in separate containers using distilled water:

KH_2PO_4 (68.07 g. per liter)
 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (123.25 g. per liter)
 $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ (109.54 g. per liter)
 NaNO_3 (42.50 g. per liter)

In preparing the macronutrient solution, add the macronutrient from the stock solutions in the following amounts to 50 liters of distilled water:

156 ml. of $\frac{1}{2}$ -molar solution of KH_2PO_4
 581 ml. of $\frac{1}{2}$ -molar solution of MgSO_4
 $\cdot 7\text{H}_2\text{O}$
 306 ml. of $\frac{1}{2}$ -molar solution of CaCl_2
 $\cdot 6\text{H}_2\text{O}$
 375 ml. of $\frac{1}{2}$ -molar solution of NaNO_3

Prepare micronutrient stock solutions:

A. Add the following micronutrients to 2 liters of distilled water:

572 g. of H_3BO_3
 3.62 g. of MnCl_2
 0.44 g. of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$
 0.16 g. of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$
 0.18 g. of H_2MoO_4

Add 1 cc. of the micronutrient stock solution per liter of prepared macronutrient solution.

B. Add 1 ml. of 0.5% ferric citrate per liter of macronutrient solution.

POTASSIUM PHOSPHATE BUFFER MIXTURES

Mixtures of potassium monohydrogen phosphate and potassium dihydrogen phosphate required to give various pH values

Volume of 0.1 molar K_2HPO_4 solution ¹	Volume of 0.1 molar KH_2PO_4 solution ²	pH of mixture	Volume of 0.1 molar K_2HPO_4 solution ¹	Volume of 0.1 molar KH_2PO_4 solution ²	pH of mixture
<i>ML.</i>	<i>ML.</i>		<i>ML.</i>	<i>ML.</i>	
10.00	—	8.302	4.00	6.00	6.643
9.90	0.10	8.171	3.00	7.00	6.468
9.75	0.25	8.038	2.00	8.00	6.239
9.50	0.50	7.863	1.00	9.00	5.910
9.00	1.00	7.648	0.50	9.50	5.600
8.00	2.00	7.347	0.25	9.75	5.303
7.00	3.00	7.146	0.10	9.90	4.976
6.00	4.00	6.976	—	10.00	4.529
5.00	5.00	6.813			

¹ 0.1 M — K_2HPO_4 = 17.4 g. dissolved in water and diluted with water to 1 liter.

² 0.1 M — KH_2PO_4 = 13.6 g. dissolved in water and diluted with water to 1 liter.

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